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United States Patent **5,653,997**
Renimel , et al. **August 5, 1997**

Antiallergic cosmetic or pharmaceutical composition

Abstract

The invention relates to the use of cucurbitine or extracts of Cucurbitaceae pips for the preparation of a cosmetic or pharmaceutical, in particular dermatological, composition having antiallergic activity, or for the preparation of cosmetic or pharmaceutical compositions having a reduced risk of being allergenic.

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Appl. No.: **476557**
Filed: **June 7, 1995**

Foreign Application Priority Data

Feb 28, 1991[FR] 91 02420

Current U.S. Class: **424/450**; 424/59; 424/70.6; 424/401; 424/758; 514/359; 514/844; 514/880; 514/881
Intern'l Class: A61K 009/127; A61K 031/40; A61K 035/78
Field of Search: 424/450,195.1,401,70,59 514/359,844,845,846,880,881

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Parent Case Text

This is a continuation of application Ser. No. 08/108,601 filed Sep. 28, 1993, now abandoned.

Claims

We claim:

1. A cosmetic or pharmaceutical composition comprising from 0.001% to 10% by weight of a cucurbitine component selected from the group consisting of cucurbitine, a cucurbitine salt and a cucurbitine ester, said cucurbitine component being encapsulated within liposome vesicles, in a cosmetically or pharmaceutically acceptable vehicle.

2. The composition of claim 1 wherein said cucurbitine component is present in laevorotatory form.

3. A pharmaceutical preparation effective for treatment of allergic manifestations, comprising approximately 100 mg of Cucurbitine, present in a pharmaceutically acceptable carrier.

4. A powdered aerosol pharmaceutical preparation effective for treatment of respiratory allergic manifestations, which comprises about 3 parts by weight, per 100 parts by weight of the preparation, of Cucurbitine in a pressurized vessel.

5. A hypoallergenic cosmetic composition for topical application to the skin or hair of a person in need thereof comprising from about 1 to about 10 parts by weight of Cucurbitine and from 99 to 90 parts by weight of one or more cosmetically acceptable additives selected from fatty alcohols, fatty esters, polyethoxylated fatty alcohols, mineral oil, humectants, gelling agents, thickeners, coloring agents, sunscreen agents and fragrance.

6. Liposome vesicles having encapsulated therein a cucurbitine component selected from the group consisting of cucurbitine, cucurbitine salt and cucurbitine ester.

7. The liposome vesicles according to claim 6 wherein said cucurbitine component is present as an extract of a ***Cucurbitaceae*** selected from the group consisting of ***Cucurbitaceae*** pips and ***Cucurbitaceae*** pulp.

8. The liposome vesicles according to claim 7 wherein said ***Cucurbitaceae*** is selected from the group consisting of Cucurbita maximum Duch, Cucurbita pepo L. and Cucurbita moschata.

9. The liposome vesicles according to claim 6 wherein said cucurbitine component is selected from the group consisting of cucurbitine, cucurbitine monohydrobromide, cucurbitine dihydrobromide, cucurbitine monohydrochloride, cucurbitine dihydrochloride, cucurbitine methyl ester and cucurbitine ethyl ester.

10. The liposome vesicles according to claim 6 wherein said cucurbitine component is present in laevorotatory form.

11. The composition of claim 1 wherein said cucurbitine component is present as an extract of a ***Cucurbitaceae*** selected from the group consisting of ***Cucurbitaceae*** pips and ***Cucurbitaceae*** pulp.

12. The composition of claim 11 wherein said ***Cucurbitaceae*** is selected from the group consisting of Cucurbita maximum Duch, Cucurbita pepo L. and Cucurbita moschata.

13. The composition of claim 1 wherein said cucurbitine component is selected from the group consisting of cucurbitine, cucurbitine monohydrobromide, cucurbitine dihydrobromide, cucurbitine monohydrochloride, cucurbitine dihydrochloride, cucurbitine methyl ester and cucurbitine ethyl ester.

Description

The present invention relates essentially to the use of cucurbitine for the preparation of an antiallergic cosmetic or pharmaceutical, in particular dermatological, compositions, and to a process involving application thereof.

Cucurbitine, or 3-amino-3-pyrrolidinecarboxylic acid, of the following formula I: ##STR1## is a natural, water-soluble amino acid found in Cucurbitaceae (see V. H. Mihranian et al., LLOYDIA (1968), 31 (1) 23-29).

Cucurbitine is known as an antiparasitic, especially as an anthelmintic against Schistosoma japonicum. (Morimoto Y. et al., Chem. Pharm. Bull. (1987) 35 (9) 3845-3849).

Cucurbitine may be obtained by means of extraction in laevorotatory form, or synthetically in racemic form. Among the various methods of synthesis of cucurbitine, special mention may be made of the synthesis method of H. J. Monteiro, J. Chem. Soc., Chem. Commun. (1973) 2. This method leads to only relatively low yields of racemic cucurbitine. Another synthesis method enables the two optical isomers of cucurbitine to be obtained separately. This is the method of Morimoto et al., Chem. Pharm. Bull. (1987) 35 (9) 3845-3849, which is a stereospecific enzymatic method of synthesis by the use of a pig liver esterase. This method is, however, complicated, and necessitates a relatively large number of steps.

It has now been discovered, unexpectedly, that cucurbitine inhibits the formation of histamine, a well-known mediator of allergies, and hence displays valuable hypohistaminaemic activity. This hypohistaminaemic activity results from the inhibitory action of cucurbitine on histidine decarboxylase, which is the enzyme responsible for the conversion of histidine to histamine. As a result, the administration of cucurbitine contributes to decreasing the histamine concentration in the blood serum and tissues.

This constitutes a considerable technical advance, since allergic manifestations, in particular pulmonary and cutaneous allergies, are nowadays causing many problems for therapists, who have a limited number of active substances at their disposal and, in addition, some of these substances can display side-effects. Thus, a considerable need consequently exists for the development of a new preventive and curative composition for allergies.

Thus, the main objective of the present invention is to solve the technical problem that consists in providing an approach enabling the formation of histamine, a mediating agent in the context of allergic manifestations, to be inhibited in order to enable allergic manifestations to be prevented and treated.

According to another aspect, the main objective of the present invention is to solve the technical problem that consists in providing an approach enabling allergic manifestations to be prevented and treated.

The object of the present invention is also to solve the new technical problem that consists in providing an approach enabling the allergenic potential of cosmetic or pharmaceutical, in particular dermatological, compositions to be decreased.

The object of the present invention is also to solve the new technical problem that consists in providing an approach enabling the synthesis of cucurbitine to be carried out by a simple synthesis process, necessitating a minimum number of steps, in good yields.

The present invention enables all these technical problems to be solved simply, reliably and reproducibly in a manner which can be used on an industrial scale.

The invention will now be described in greater detail with reference to the following description and preferred embodiments and with the assistance of the accompanying drawings.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 is a Mickaelis curve of the inhibitory activity with respect to enzyme histidine decarboxylase (HDC) for each of control, tritoqualine and cucurbitine in terms of initial rate versus amount histidine; and

FIG. 2 is a graphic plot of an RIA assay for antihistaminic activity of cucurbitine, according to this invention, in comparison to that of tritoqualine and a control.

Thus, according to a first aspect, the present invention relates to the use of cucurbitine or one of its of a cosmetic or pharmaceutical, in particular dermatological, composition having antiallergic activity.

According to a particular variant of embodiment, the preparation in question is of a cosmetic or pharmaceutical, in particular dermatological, composition intended for the prevention or symptomatic treatment of allergic manifestations, irrespective of their origin and their point of application, in particular the bronchi, skin and eye. Thus, the said composition is intended, in particular, for the prevention or symptomatic treatment of allergic or exercise-induced bronchial asthma, hay-fever, spasmodic tracheitis and rhinitis, urticaria, other allergic eruptions, eczema, red blotches or skin irritations of allergic origin, pruritus, Quincke's oedema, allergic conjunctivitis and also allergic reactions of medicinal origin.

In the more particular field of cosmetology, the said composition is intended advantageously for lines of products which are hypoallergenic or for sensitive or irritable skins.

Cucurbitine may be used either in free form, or in the form of one of its cosmetically or pharmaceutically, in particular dermatologically, acceptable salts or esters. The abovementioned salts and esters may be prepared by conventional processes which are well known to a person skilled in the art. Among salts, the mono- and dihydrobromide and the mono- and dihydrochloride may be mentioned. Among esters, the methyl ester and the ethyl ester may be mentioned.

According to an advantageous variant, the abovementioned plant extract containing cucurbitine is an extract of Cucurbitaceae, especially of Cucurbita maxima Duch., of Cucurbita pepo L. or of Cucurbita moschata Duch; preferably, it is an extract of pips or of fruit pulp. As a further preference, it is an extract of Cucurbitaceae pips.

According to a particular variant, the abovementioned plant extract is an extract of Cucurbitaceae fruit pulp containing at least 0.5% by weight of curcubitine.

According to an advantageous variant of embodiment, cucurbitine or one of its cosmetically or pharmaceutically, in particular dermatologically, acceptable salts or esters is present at a concentration of 0.001% to 10%, and preferably 0.01 to 5%, by weight of the total composition.

According to a second aspect, the present invention also relates to a cosmetic composition, characterized in that it comprises, as active ingredient, cucurbitine or one of its cosmetically acceptable salts or esters, or a plant extract containing it, where appropriate in a cosmetically acceptable excipient, vehicle or carrier.

According to an advantageous variant, the abovementioned plant extract is an extract of Cucurbitaceae as defined above.

According to a preferred variant of embodiment, cucurbitine or one of its salts or esters is present in an amount which is effective for displaying antiallergic activity, especially at a concentration of 0.001% to 10%, and preferably 0.01% to 5%, by weight of the total composition.

According to a third aspect, the present invention relates to a pharmaceutical, in particular dermatological, composition preferably having antiallergic activity, characterized in that it contains cucurbitine or one of its pharmaceutically acceptable salts or esters in an amount which is effective for displaying antiallergic activity, especially for preventing or treating allergic manifestations, in particular in the bronchi, skin and eye, where appropriate in a pharmaceutically acceptable excipient, vehicle or carrier.

According to a variant, the abovementioned cosmetic and pharmaceutical compositions contain an extract of plant origin as defined above.

According to a fourth aspect, the present invention further relates to a process for decreasing the allergenic potential of a cosmetic or pharmaceutical, in particular dermatological, composition, characterized in that an effective amount of cucurbitine, in free form or in the form of one of its cosmetically or pharmaceutically acceptable salts or esters, or of a plant extract containing it as defined above, is incorporated in the said composition so that the final composition presents a reduced risk of being allergenic.

In the context of any one of the above aspects, the preferred weight concentration of cucurbitine, or of its salts or esters, for compositions for topical use, is between 0.001% and 10%, and as a further preference between 0.01% and 5%.

For compositions intended for administration systemically (such as orally, parenterally, rectally, by inhalation, etc.), the cucurbitine concentration is not critical and can reach, for example, 60% of the composition. The dosage in man will generally be between 0.1 mg/kg/day and 20 mg/kg/day, and preferably between 1 mg/kg/day and 15 mg/kg/day.

Moreover, natural cucurbitine, of laevorotatory form, or its salts or esters, is/are generally used. Especially advantageous sources of laevorotatory cucurbitine are the pulp and pips of Cucurbitaceae, especially of the species Cucurbita pepo L., Cucurbita maxima Duch. and Cucurbita moschata Duch. However, the racemic form of cucurbitine or of its salts or esters may also be used.

Moreover, cucurbitine may be used in pure form, or in the form of extracts, according to any one of the extraction procedures known to a person skilled in the art. Especially advantageous extraction procedures are described in the publication of Valentine H. MIHRANIAN et al. in lloydia 1968, 31, (1) 23-29. This process advantageously provides for treatment of decorticated and defatted Cucurbitaceae seeds with water, which is advantageously heated to at least 50.degree. C. with constant stirring for several hours. Thereafter the mixture is centrifuged, the supernatant is collected and the residues are re-extracted one or more times in a similar manner with several portions of heated water. The supernatant and washings are combined and then treated by adding to an equivalent volume of 85% ethanol to precipitate proteinaceous matter in suspension, and the whole is kept in a refrigerator overnight. The mixture is then centrifuged and the supernatant is collected. The alcohol may be removed by distillation, for example in a rotary evaporator. The aqueous solution may be used as it is, or alternatively passed thereafter through a chromatography column for example of the

Dowex 50W-X-8 type, for example measuring 75.times.2.2 cm. The column is washed with approximately 200 ml of water, and then eluted with 1% aqueous ammonium hydroxide solution until the effluent gives a negative response to the ninhydrin test. The eluate may be evaporated to dryness under reduced pressure, heating where appropriate. The syrupy residue may, again, be used as it is, or alternatively treated again with hot water and advantageously added to an at least equivalent volume of 95% ethanol. Thereafter, the mixture may be acidified dropwise with an acid to bring the pH of the solution to a pH approximately equal to 5, for example with 60% perchloric acid. It may then be placed in a refrigerator for several days to obtain a precipitate of cucurbitine perchlorate. This precipitate can then be dissolved in a few milliliters of water and, where appropriate, passed through a chromatography column, preferably of the Amberlite CG-45 type, for example measuring 20.times.1.5 cm. Evaporation of the eluate under reduced pressure gives cucurbitine in the substantially pure state.

Thus, the above process makes it possible to obtain either cucurbitine in the pure state, or extracts having variable cucurbitine contents. In the case where cucurbitine is used in the form of an extract of Cucurbitaceae, the cucurbitine content is preferably equal to at least 0.5% by weight of the extract. A preferred source for obtaining cucurbitine consists of the pips of the Cucurbita species such as Cucurbita pepo L., Cucurbita maxima Duch. and Cucurbita moschata Duch.

According to a fifth aspect, the present invention also covers a process for the treatment of a human being or an animal for preventing or treating allergic manifestations, characterized in that an amount of cucurbitine or one of its pharmaceutically acceptable salts or esters, or of a plant extract containing it as defined above, which is effective for preventing or treating allergic manifestations is administered to the said human being or said animal.

In particular, the abovementioned treatment is applied to the prevention or symptomatic treatment of allergic or exercise-induced bronchial asthma, hayfever, spasmodic tracheitis and rhinitis, urticaria and other allergic eruptions, eczema, red blotches or skin irritations of allergic origin, pruritus, Quincke's oedema, allergic conjunctivitis and also allergic reactions of medicinal origin.

According to a variant of embodiment, cucurbitine or one of its pharmaceutically acceptable salts or esters is administered topically at a concentration preferably of between 0.001% and 10% by weight.

According to another variant of embodiment, cucurbitine or one of its pharmaceutically acceptable salts or esters is administered systemically at a dosage in man of between 0.1 mg/kg/day and 20 mg/kg/day, and preferably between 1 mg/kg/day and 15 mg/kg/day.

The invention further relates to a process for preparing a cosmetic or pharmaceutical, in particular dermatological, composition, characterized in that cucurbitine or one of its cosmetically or pharmaceutically acceptable salts or esters, or a plant extract containing it as defined above, is incorporated in a cosmetically or pharmaceutically acceptable carrier, vehicle or excipient.

According to a variant of embodiment, cucurbitine or one of its cosmetically or pharmaceutically acceptable salts or esters is incorporated in a complete cosmetic or pharmaceutical formulation for decreasing the risk of the latter being allergenic.

According to another variant of embodiment, a composition having antiallergic activity is prepared. Some variants of preparation also result from the foregoing description.

According to a particular embodiment of the invention in the context of any one of the aspects stated

above, the abovementioned composition containing cucurbitine or one of its salts or esters, or the abovementioned plant extract, contains, in addition, vesicles of the liposome type. According to a particular variant, the cucurbitine, its salt or ester is at least partially encapsulated in vesicles of the liposome type. The expression "vesicle of the liposome type" is understood to mean both hydrated lamellar lipid phases and lipid vesicles composed of ionic or nonionic amphiphilic lipids. Also, the expression "to incorporate at least partially in vesicles of the liposome type" is understood, in the present description and the claims, to mean that the cucurbitine, its salt or ester is combined with vesicles of the liposome type irrespective of the form of this combination. However, a preferred combination lies in encapsulation of the cucurbitine, its salt or ester in vesicles of the liposome type. However, it is not necessary for the total amount to be incorporated or encapsulated in order to obtain the desired antiallergic effect according to the invention.

It is known that vesicles of the "liposome" type are prepared from lipid substances. The term "lipid" covers all substances comprising a so-called fatty carbon chain, generally containing more than 5 carbon atoms, this substance customarily being designated "lipid".

According to the invention, to form either the lamellar lipid phases or the vesicles of the liposome type, amphiphilic lipids, that is to say lipids consisting of molecules possessing a hydrophilic group which can be equally well ionic or nonionic and a lipophilic group, are used as lipids, these amphiphilic lipids being capable of forming lamellar lipid phases or vesicles of the liposome type in the presence of an aqueous phase.

In particular, among these lipids, there may be mentioned: phospholipids, phosphoaminolipids, glycolipids, polyoxyethylenated fatty alcohols and optionally polyoxyethylenated esters of a polyol. Such substances consist, for example, of an optionally hydrogenated egg or soya bean lecithin, a phosphatidylcholine, a phosphatidylserine, a sphyngomyelin, a cerebroside or an oxyethylenated polyglycerol stearate.

Incorporation of the compounds used according to the present invention in hydrated lamellar lipid phases or in liposomes may be carried out according to known preparation techniques, described, for example, in the document U.S. Pat. No. 4,508,703, and, where appropriate, in combination with the document U.S. Pat. No. 4,621,023.

According to a seventh aspect, the present invention also covers a process for the synthesis of cucurbitine, characterized in that 1-benzyl-3-pyrrolidinone is used as starting material.

According to a particular variant of embodiment of this synthesis process, 1-benzyl-3-pyrrolidinone is treated with an ammoniacal solution of ammonium chloride and of potassium cyanide to obtain (.+-.)-3-amino-1-benzyl-3-cyanopyrrolidine. This compound is then converted by acid or basic hydrolysis to (.+-.)-3-amino-1-benzyl-3-pyrrolidinecarboxylic acid, and lastly a reduction with hydrogen, preferably a catalytic hydrogenolysis, is carried out to obtain (.+-.)-3-amino-3-pyrrolidinecarboxylic acid or (.+-.)-cucurbitine.

According to a preferred variant, the abovementioned ammoniacal solution is an aqueous-alcoholic solution, the alcohol advantageously being isopropanol or methanol.

According to another preferred variant, the abovementioned hydrolysis is performed using 6N aqueous hydrobromic acid solution.

According to yet another variant, the catalytic hydrogenolysis is performed in water under hydrogen

at atmospheric pressure in the presence of a catalyst such as palladium on charcoal dispersed in the aqueous reaction medium.

According to a preferred embodiment of the synthesis process, 1-benzyl-3-pyrrolidinone is allowed to react with ammonium chloride and potassium cyanide in a 1:4:4 mole ratio at room temperature for at least 48 h.

According to another particular variant of embodiment, the optical isomers are separated from the racemic mixture according to any separation technique known to a person skilled in the art, and especially via the preparation of diastereoisomers.

The process for the synthesis of cucurbitine according to the invention leads to especially high yields of racemic cucurbitine, on average 2 to 3 times as high as those of the known processes.

Other objects, features and advantages of the present invention will become clearly apparent in the light of the explanatory description which follows and which is given with reference to various examples of preparation of cucurbitine, as well as to various examples reporting the results of pharmacological tests, as well as various examples of cosmetic or pharmaceutical formulation. In the examples, the percentages are given by weight except where otherwise stated.

EXAMPLE 1

Synthesis of cucurbitine in the form of a racemic mixture

The procedure is as follows:

a) Synthesis of (.+-.)-3-amino-1-benzyl-3-cyanopyrrolidine

0.5 g of 1-benzyl-3-pyrrolidinone (2.85 mmol) dissolved in 3 ml of 2-propanol is added to a solution of 0.741 g (11.4 mmol) of potassium cyanide and 0.615 g (11.4 mmol) of ammonium chloride in 7 ml of 28% ammonia solution. The mixture remains at room temperature with stirring for 3 days. The solution is washed with 15 ml of 10% potassium carbonate solution and extracted with dichloromethane (3.times.c 15 ml). After drying over magnesium sulphate and evaporation of the solvents, an oil (0.475 g) is obtained.

The product is purified on a silica column with solid loading. It is eluted with a 4:2 ether/petroleum ether mixture.

0.402 g of a beige solid is obtained (yield: 70%), the solid consisting of 3-amino-1-benzyl-3-cyanopyrrolidine having the following NMR spectrum:

.sup.1 HNMR, 300 MHz, CDCl.sub.3 1.8 (broad s, 2H, NH.sub.2); 1.97 (ddd, 1H, J.sub.4,4' =13.4, J.sub.4,5 =8, J.sub.4,5' =5.4); 2.5 (ddd, 1H, J.sub.4,4' =8, J.sub.4,5' =qJ.sub.4',5' =5.4); 2.64 (d, 1H, J.sub.2,2' =9.4); 3.04 (d, 1H, J.sub.2',2 =9.4); 3.67 (s, 2H, CH.sub.2 --C.sub.6 H.sub.5); 7.33 (m, 5H, aromatic protons).

b) Synthesis of (.+-.)-3-amino-1-benzyl-3-pyrrolidinecarboxylic acid or (.+-.)-1-benzylcucurbitine

The hydrolysis of the compound obtained above may be carried out either in an acid medium or in a basic medium.

Hydrolysis in an acid medium:

0.3 g (1.49 mmol) of 3-amino-1-benzyl-3-cyanopyrrolidine obtained in step a), dissolved in 5 ml of 48% hydrobromic acid, are brought to 40.degree.-50.degree. C. for 4 h. After evaporation of the acid, the product is purified on a silica column. The impurities are removed with CH.sub.2 Cl.sub.2 /MeOH 10% and amino acid is brought off with MeOH/H.sub.2 O 15%.

After removal of the methanol, the compound is decolorized with animal charcoal in a minimum amount of aqueous medium in the heated state and then lyophilized.

A dark yellow solid is obtained (yield: 80%), consisting of (.+-.)-3-amino-1-benzyl-3-pyrrolidinecarboxylic acid in the form of a mono- or dihydrobromide having the following NMR spectrum:

.sup.1 HNMR, 300 MHz, D.sub.2 O 2.47-2.69 (m, 1H, H.sub.4); 2.75-2.91 (m, 1H, H.sub.4 '; 3.69-3.94 (m, 3H, H.sub.5, H.sub.5', H.sub.2); 4.12 (d, J.sub.2,2' =14.2, 1H, H.sub.2'); 4.58 (2d, J=13.8, 2H, CH.sub.2 --C.sub.6 H.sub.5); 7.62 (s, 5H, aromatic protons).

Hydrolysis in a basic medium:

280 mg (1.39 mmol) of 3-amino-1-benzyl-3-cyanopyrrolidine of step a) are dissolved in 2 ml of ethanol. 5 ml of 10% sodium hydroxide solution are added and the mixture is then brought to reflux for 5 h. After cooling, it is acidified with 48% hydrobromic acid.

The product is purified on a silica column: MeOH/H.sub.2 O 10%. The product is decolorized with animal charcoal and then lyophilized. The presence of sodium bromide salts causes a yield of greater than 100% to be obtained.

To remove the salts present, 200 mg of pyrrolidine are taken and brought to pH 8 with 10% sodium hydroxide solution. Duolite Cl.sup.- is loaded with 2N hydrobromic acid solution. The salts are removed with distilled water. To detach the pyrrolidine, 0.1N hydrobromic acid solution is used.

After lyophilization, 120 mg of (.+-.)-3-amino-1-benzyl-3-pyrrolidinecarboxylic acid are obtained (yield: 40%).

c) Synthesis of (.+-.)-3-amino-3-pyrrolidinecarboxylic acid or (.+-.)-cucurbitine

300 mg of (.+-.)-3-amino-1-benzyl-3-pyrrolidinecarboxylic acid hydrobromide obtained in step b) are dissolved in 10 ml of water. 0.5 mg of charcoal obtaining 10% of palladium is dispersed, and this suspension is then placed in a hydrogen atmosphere at atmospheric pressure. Stirring is maintained for 18 h. After filtration through filter paper and lyophilization, a yellow solid is obtained (98%), consisting of (.+-.)-3-amino-3-pyrrolidinecarboxylic acid or (.+-.)-cucurbitine having the following NMR spectrum:

.sup.1 HNMR, 300 MHz, D.sub.2 O 2.45 (m, 1H, H.sub.4); 2.69 (m, 1H, H.sub.4'); 3.61 (d, 1H, J.sub.2,2' =13.4); 3.67-3.78 (m, 2H, H.sub.5, H.sub.5'); 4 (d, 1H, J.sub.2',2 =13.4).

EXAMPLE 2

Optimization of the yield of the synthesis of (.+-.)-cucurbitine

The procedure is as described in Example 1, choosing in step b) hydrolysis with 6N hydrobromic acid, varying, however, the proportion of the reactants of step a).

The yields obtained appear in Table 1 below.

TABLE I

Number of equivalents Moles N.B.P.	NH.sub.4 Cl	KCN	Experimental conditions of step a)	Yield CN	COOH
1	1--1	1	6h room temperature	*	<10%
1	1--1	1	4 h 40.degree.-50.degree.	*	<10%
1	4	4	4 h 40.degree.-50.degree.	*	20%
1	8	8	4 h 40.degree.-50.degree.	*	20%
1	4	4	48h room temperature	66%	44%
1	4	4	72h room temperature	70%	49%
1	4	4	84h room temperature	70%	56%

N.B.P.: 1benzyl-3-pyrrolidinone
*: aminonitrile, not isolated
CN: yield of 3amino-1-benzyl-3-cyanopyrrolidine
COOH--: overall yield of 3amino-3-pyrrolidinecarboxylic acid

The last column of Table I contains the yields of (.+-.)-cucurbitine relative to the starting reactants.

It is observed that the yields are maximal if the reaction is carried out at room temperature for a period exceeding 48 h, using proportions of 1:4:4 between the reactants: 1-benzyl-3-pyrrolidone, ammonium chloride and potassium cyanide.

It was, moreover, observed that hydrolysis by means of hydrobromic acid also led to better yields than when hydrochloric acid at the same concentration is used as hydrolysis agent.

EXAMPLE 3

Separation of the optical isomers of cucurbitine

The separation method, which is known per se, is based on the preparation of diastereoisomeric derivatives by coupling of (.+-.)-cucurbitine with certain optically active reagents after protection of the acid function by esterification and of the cyclic amine function. Each form of these stereoisomers, corresponding to one or other isomer of cucurbitine, will be isolated by chromatography, in particular by high performance liquid chromatography or on a silica column. To regenerate thereafter the two

enantiomers of cucurbitine, it suffices to saponify the protecting ester, then to hydrolyse it in order to liberate the acid function, and to liberate the amine function, for example by hydrogenolysis as described in Example 1 in the case where the amine function was protected by substitution with a benzyl radical.

As reagents for the present method, it is possible to use laevorotatory S optical active compounds such as:

(1S)-camphanyl chloride,

(S)-(-)-.alpha.-methoxy-.alpha.-(trifluoromethyl)phenylacetic acid,

N-(tert-butoxycarbonyl)-L-phenylalanine.

The present example describes the resolution of the racemate of cucurbitine by means of coupling with (1S)-camphanyl chloride.

a) Protection of the acid function of (+)-1-benzylcucurbitine: synthesis of its methyl ester

0.1 g of (.+-.)-1-benzylcucurbitine (0.20 mmol), obtained in step b of Example 1, is added slowly to an ice-cold solution of thionyl chloride (0.28 mmol) and methanol (2 ml). The reaction temperature must not rise above -5.degree. C. The mixture is stirred at 0.degree. C. for 2 h and then brought back to room temperature for two days.

After evaporation, the product is purified on a silica column with solid loading. (Eluent CH.sub.2 Cl.sub.2 /MeOH from 10 to 50%).

A yellow product is obtained in a 70% yield, consisting of (.+-.)-1-benzylcucurbitine methyl ester.

b) Coupling with (1S)-(-)-camphanyl chloride

1 mol of (.+-.)-1-benzylcucurbitine methyl ester, dissolved in 1.5 ml of methylene chloride, is neutralized with 1 mol of triethylamine. 1.1 equivalents of (1S)-(-)-camphanyl chloride are added. The mixture is left stirring at room temperature for 16 h.

The product is purified on a silica column with solid loading (eluent: CH.sub.2 Cl.sub.2 /MeOH 10%).

Separation of the diastereoisomers was performed by HPLC on various columns, in particular NH.sub.2 -grafted Zorbax.RTM..

EXAMPLE 4

Production of cucurbitine from Cucurbita pepo pulp

Fresh Cucurbita pepo fruits are cut in half, and the pips which can be used for the manufacture of pip extracts are removed. The pulp thereby obtained is ground and lyophilized. The powder is recovered and defatted with petroleum ether in the proportion of 1 l to 100 g of powder. The insoluble matter, which constitutes the desired extract of Cucurbita pepo pulp, is recovered by filtration. The proportion of cucurbitine in this extract is assayed by HPLC, and a concentration of 0.03% by weight

of cucurbitine is obtained in the defatted dry extract.

The defatted dry extract is then introduced into water, which is heated to approximately 50.degree. C. with stirring until dissolution is complete. An equivalent volume of 95% ethanol is added to precipitate proteinaceous matter in suspension, which is removed by centrifugation. The clear supernatant remaining is then acidified to pH 5.0 with 60% perchloric acid. The solution thus acidified is placed in a refrigerator for at least 2 days to precipitate the cucurbitine perchlorate, which is collected. Cucurbitine may be obtained from this cucurbitine perchlorate in a conventional manner for a person skilled in the art, especially by passage through a cation exchange resin column (Na.sup.+ type) and by evaporation of the eluate under reduced pressure.

EXAMPLE 5

Preparation of an extract of Cucurbita pepo seeds

1.5 kg of previously decorticated Cucurbita pepo seeds are ground. The powder obtained is subjected three times to extraction with hexane (3 liters, 2 liters and 1.8 liters) to remove fats. The dried cake obtained is extracted with aqueous hydrochloric acid solution maintained at approximately pH 4. This extraction is carried out in three stages: two at room temperature--approximately 22.degree. C.--and the third at 70.degree. C. At each stage, the time during which the cake-solution is kept in contact with the hydrochloric acid solution (2 liters on the first occasion and 2.5 liters on the following two occasions) is 24 h.

After draining, the solid residue is removed and the aqueous phase is collected, partially evaporated and centrifuged. The centrifugation pellets are washed with distilled water and then discarded. The washing liquors are combined with the centrifugation concentrates.

The aqueous fraction is reconcentrated and then treated with an equal weight of ethanol. A white precipitate forms, which is removed by centrifugation. The aqueous-alcoholic supernatant, which is neutralized, for example, with sodium hydroxide, constitutes an extract containing (-)-cucurbitine, which may be used as it is.

It is also possible to evaporate off the alcohol and then to atomize the aqueous solution obtained, so as to obtain a powder assaying at 3 to 5% of (-)-cucurbitine, depending on the batches of seeds used.

EXAMPLE 6

Preparation of a (-)-cucurbitine-rich extract of Cucurbita pepo seeds, and production of purified (.+-.)-cucurbitine.

A--Preparation of an extract of cucurbita pepo seeds

The starting material consists either of Cucurbita seeds, decorticated or otherwise, having a (-)-cucurbitine content of 0.2 to 0.4 percent by weight, depending on the batch or origin, or of oil-free cakes of Cucurbita seeds having a (-)-cucurbitine content of 0.4 to 0.8 percent by weight, depending on the batch or origin.

The starting material is preferably ground to approximately 100 micrometers. The ground material is then treated by soaking at room temperature with acidulated water (sulphuric acid at a concentration of 0.1 percent by weight) at pH 3.5 approximately, for 2 h in the case of ground cakes or up to 16 h in

the case of seeds. The amount of acidulated water used is approximately five times the weight of the starting material. The whole is then brought to boiling at atmospheric pressure for 1 h. After cooling to 80.degree. C., the mixture is filtered and then, where appropriate, centrifuged. The aqueous phase obtained is then brought to a temperature of 80.degree. C. to 90.degree. C. and thereafter microfiltered through a 0.5 micrometer filter. A juice assaying at approximately 10 g of dry matter per liter is thereby obtained.

This juice then undergoes a thermal preconcentration under a partial vacuum to a concentration of approximately 250 g/1 of dry matter. This preconcentrate is thereafter placed at 4.degree. C. for 48 h, then undergoes a further concentration under reduced pressure to 500 g of dry matter per liter, and is again left standing at 4.degree. C. for 48 h. These operations are followed by filtration through a filter press. An extract containing from 40 to 50% of dry matter and assaying at between 1 and 2% of (-)-cucurbitine is thereby obtained. The yields are approximately 200 l of extract per tonne of starting material.

B--Production of purified (-)-cucurbitine

The abovementioned preconcentrate, assaying at 250 g to 300 g of dry matter per liter, is neutralized with sodium hydroxide to obtain a pH of 7.5. The mixture is filtered through a filter press and the filtrate is then passed through a cation exchange resin (of the Na.sup.+ type). Elution is performed using ammonia solution. After thermal concentration of the eluate under a partial vacuum, a syrup is obtained, the dry matter of which contains approximately 50% by weight of (-)-cucurbitine. If so desired, this syrup may by lyophilized. For this purpose, it will advantageously be mixed with a neutral powdery carrier such as talc.

EXAMPLE 7

Demonstration of the inhibitory activity of cucurbitine with respect to histamine formation

1--A by an enzymatic test

This test is based on the inhibitory action of cucurbitine on the enzyme histidine decarboxylase (HDC), which converts histidine to histamine, in comparison with that of tritoqualine, which is a known inhibitor of HDC (see Carpi C., Maggi G. C. Bull. Soc Ital. Sper. 1968, 44 (6 543-4) and which is used therapeutically as a hypohistaminaemic agent under the name Hypostamine.RTM..

The inhibitory activity with respect to HDC may be readily assessed by a colorimetric assay on the basis of the following chemical reaction: ##STR2##

In practice, it is observed that the formation of the blue coloration is proportional to the concentration of histidine consumed. It has thus been possible to define initial rates of reaction and to plot so-called Mickaelis curves as described in Fundamentals of Enzymology, 2nd Ed. Oxford Univ. Press, 1989.

The initial rate is expressed as a change in absorbance per minute.

The results obtained with various initial concentrations of histidine, namely 132 nmol, 265 nmol and nmol, respectively without effector, with tritoqualine as a known HDC inhibitor and with cucurbitine in racemic form as inhibitor according to the invention, are recorded in Table II below, and form the subject of the Mickaelis curve which is the subject of FIG. 1.

TABLE II

Histidine	132 nmol	265 nmol	530 nmol
Without effector	0.062	0.089	0.1132
Tritoqualine (prior art)	0.021	0.0374	0.0604
Cucurbitine (invention)	0.013	0.0242	0.042

It emerges clearly from Table II and from the Mickaelis curve which is the subject of FIG. 1 that cucurbitine is a much more potent HDC inhibitor than tritoqualine, constituting an altogether surprising result for a person skilled in the art.

1--B by a RIA assay

The antihistaminic activity of cucurbitine may also be demonstrated by a radioimmunological assay (Radio Immuno Assay or "RIA") in the following manner.

This assay takes place by assaying the histamine produced directly under the action of the enzyme HDC, by the RIA method which is well known to a person skilled in the art and described, in particular, in the directions for use of an assay kit called HISTAMINE Radioimmunoassay kit (Cat. #1302) marketed by the company IMMUNOTECH (Marseilles-France).

The amount of histamine liberated (in nanomoles) for a 16.times.10.sup.-3 molar concentration of histidine in a phosphate buffer at pH 6.3 is assayed over time, respectively without effector, with tritoqualine as effector for comparison and with synthetic racemic cucurbitine as antihistaminic agent according to the invention. Tritoqualine and cucurbitine are used at a concentration of 2.times.10.sup.-3 molar.

The results obtained, expressed as nanomoles of histidine liberated, are recorded in Table III below and form the subject of the curve of FIG. 2, where the number of nanomoles of histamine liberated has been shown as ordinates and the time expressed in minutes as abscissae. The curve without effector is plotted as a continuous line, the curve obtained with tritoqualine is plotted as a chain-dotted line and the curve obtained with cucurbitine is plotted as a dotted line.

TABLE III

Time	5 min**	10 min**	% I* (at 5 min)	% I* (at 10 min)
Without effector	32.66	140.79	0	0
Tritoqualine	16.48	49.27	49	65
Cucurbitine	14.85	38.29	55	73

*I = Inhibition

** = nanomoles of histamine liberated

It is clearly seen from Table III that the amount of histamine liberated is markedly lower in the presence of cucurbitine.

The results obtained by the RIA method hence confirm that cucurbitine has a markedly more potent inhibitory activity with respect to histamine formation than tritoqualine after a few minutes.

Various examples of formulation of cosmetic or pharmaceutical, in particular dermatological, compositions according to the invention are as follows:

EXAMPLE 8

Tablets

Per tablet for oral administration:

(.+-.)-cucurbitine	100 mg
starch	38 mg
lactose	75 mg
talc	10 mg
other excipients for tablets (including magnesium stearate) qs	250 mg

Indications: preventive and curative treatment of allergic manifestations, in particular cutaneous and respiratory manifestations.

Dosage: 1 to 10 tablets per day for adults. Decrease the dosage by 2 for children up to 15 years of age.

EXAMPLE 9

Powder for aerosol

(.+-.)-cucurbitine	3 g
mannitol	1 g
propellent gases	96 g

Indications: preventive and curative treatment of all respiratory allergic manifestations such as bronchial asthma and asthmoid bronchitis.

Dosage: intrabronchial administration, at the rate of 4 to 6 inhalations per day on average.

EXAMPLE 10

Demulcent emulsion for sensitive skins

pip extract according to Example 5	10 g
assaying at 3% of (-)-cucurbitine	
conventional cosmetic emulsion	100 g
for sensitive skins (fatty alcohols, polyoxyethylenated fatty alcohols, mineral oil, isopropyl palmitate, glycerol, gelling agent, preservatives, fragrances, water) qs	

EXAMPLE 11

Hypoallergenic make-up foundation

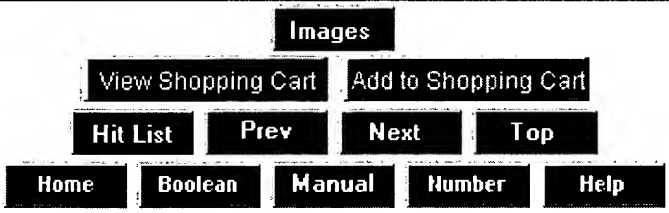
extract of Cucurbita pepo pulp,	10 g
fortified with (-)-cucurbitine, according to Example 4, having a content of 1% of (-)-cucurbitine	
conventional make-up foundation	100 g
composition (fatty esters, squalane, soya bean lecithin, volatile silicone, propylene glycol, xanthan gum, sunscreen agent, pigments, preservatives, fragrance, water) qs	

EXAMPLE 12

Anti-irritant mascara

(.+-.)-cucurbitine	1.0 g
ultramarine blue	9.0 g
hexadecyl alcohol	7.4 g
propylene glycol	9.0 g
stearic acid	11.3 g
glycerol monostearate	4.4 g
triethylamine	3.6 g
preservative	0.3 g
water qs	100 g

* * * * *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PATENT FULL TEXT AND IMAGE DATABASE

United States Patent **6,174,920**
Hicks , et al. **January 16, 2001**

Method of controlling powdery mildew infections of plants using jojoba wax

Abstract

A new method of eradicating an existing infection of powdery mildew on plants using a wax ester emulsion is provided wherein the wax ester emulsion is comprised of wax esters, which are derived from esterification of monoethylenic acids and monoethylenic alcohols having between 18 and 24 carbons, and surfactant at a concentration of between 1% v/v and 10% v/v of the total wax ester component.

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Appl. No.: **316482**
Filed: **May 21, 1999**

Current U.S. Class: **514/549**; 424/776; 514/506; 514/529; 514/546; 514/552; 514/783; 514/785; 514/937; 514/943; 514/975
Intern'l Class: A01N 037/00; A01N 025/00; A01N 061/00
Field of Search: 514/975,506,529,546,552,549,783,785,937,943 424/195.1

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Claims

What is claimed is:

1. A method of eradicating an existing infection of powdery mildew on a plant said method comprising:

spraying onto the surface of a plant with an existing infection of powder mildew an aqueous emulsion of a wax ester or mixture of wax esters in an amount effective to reduce the powdery mildew by at least 50% in the absence of the wax ester, wherein at least 50% v/v of the wax ester consists of an esterification product of monoethylenic acids having 18 to 24 carbons and monethylenic alcohols having 18 to 24 carbons, with the proviso that spraying is conducted when i) the ambient temperature is below 80 degrees Fahrenheit and is not conducive to white fly infestations and ii) the plant is not exhibiting anthesis.

2. The method of claim 1 with the further proviso that the plant is not a member of the family Vitaceae.

3. The method of claim 1 wherein the plant is an apple tree and the powder mildew is Podosphaera leucotricha.

4. The method of claim 1 wherein the plant is an eggplant and the powder mildew is Erysiphe cichoracearum.

5. The method of claim 1 wherein the plant is a strawberry plant and the powder mildew is Sphaerotheca humuli.

6. The method of claim 1 wherein the plant is a pea plant and the powder mildew is Erysiphetis.

7. The method of claim 1 wherein the plant is a ***Cucurbitaceae*** and the powder mildew is Sphearothecafuliginea.

8. The method of claim 1 wherein the plant is a tomato plant and the powdery mildew is Leveillula taurica.

9. The method of claim 1 wherein the wax ester, or wax esters are extracted from jojoba seeds.

10. The method of claim 1 wherein the emulsion further comprises surfactant other than said wax ester(s).

11. The method of claim 1 wherein the emulsion further comprises surfactant, other than said wax ester(s), that is a non-ionic detergent.

12. The method of claim 1 wherein the emulsion further comprises surfactant selected from the group consisting of: ethoxylated alkyl phenyl ethers, siloxanes and polysiloxanes.

13. The method of claim 1 wherein the emulsion further comprises a surfactant, other than said wax ester(s), present at a weight ratio of wax ester(s) to said surfactant from 1:10 to about 1: 50.

14. The method of claim 1 wherein the emulsion has a concentration of between about 0.25% to about 5% v/v wax ester(s).

15. The method of claim 1 wherein about 50% to about 90% v/v of the wax ester is a mixture of ecosenyl eicosenoate and decosenyl eicosenoate.

16. The method of claim 1 wherein the wax esters are the same in type and concentration to the wax esters found in naturally occuring jojoba extract.

Description

FIELD OF THE INVENTION

This invention relates to a method of eradicating powdery mildew infection of plants by the application of a fungicidal wax ester emulsion, wherein the wax esters are the esterification product of monoethylenic acids and monoethylenic alcohols having between 18 to 24 carbons each and are emulsified in water with surfactant. Preferably, the wax esters are comprised of a mixture of wax esters which are at least 85% identical to the type and concentration of wax ester found in naturally occurring jojoba extract, with a surfactant at a concentration between 1% v/v and 10% v/v of the total wax ester mixture.

BACKGROUND OF THE INVENTION

Over the last thirty years it has been a goal of various government agencies to develop uses for the desert plant jojoba (Simmondsia chinensis), especially the extract produced from the jojoba seeds. Due to the jojoba plant's ability to thrive in arid climatic conditions in coarse desert soils and it's life

span which can extend up to 200 years, the plant was thought to be particularly useful for developing an agricultural industry in the underutilized lands of the American southwest.

The jojoba plant produces an abundance of seeds with an average yield for a mature plant of about 12 pounds (dry weight). Jojoba seeds contain about 50% by weight of a colorless, odorless liquid extract which is commonly referred to as "jojoba oil". The extract is chemically an unsaturated wax made up of non-glyceride esters having a narrow range of chemical composition. Waxes of this type are relatively rare. Another natural source for wax esters is spermaceti, a liquid wax produced from the head of the sperm whale. As the sperm whale is an endangered species and interstate sale of its oil was banned in 1973, it is not recognized as a particularly useful source for wax esters.

Jojoba extract is more than 97% wax esters. Each wax ester is derived from one molecule of a long-chain monoethylenic alcohol esterified with one long-chain monoethylenic fatty acid. The wax esters typically are comprised of carbon chains of 38 to 44 carbon atoms. Suprisingly, the wax ester components of jojoba extract have been found to exhibit superior fungicidal capabilities.

Powdery mildew is the common name applied to one of the most damaging and wide spread diseases in the agricultural and horticultural industry. Powdery mildew is a disease caused by species of several genera of fungus on a number of different host plants. The variety of horticultural and agricultural plants susceptible to powdery mildew disease is large. The disease is called "powdery mildew" because the appearance of the mycelial growth gives a powder like appearance on the surface of the host. The white powdery growth appears on infected leaves, stems, fruit and flower buds. Infected leaves may also appear distorted and fall from the plant. Powdery mildew spores are easily spread by wind to nearby plant tissue and to other plants, resulting in an epidemic if left unchecked.

Various chemical compounds have been disclosed which claim suitability for use as a fungicide for controlling powdery mildew infection. For example, U.S. Pat. No. 5,366,995 discloses the use of short chain fatty acids and their salts as a fungicidal agent. The disclosed fatty acids generally have carbon chains of between 7 to 20 atoms and preferably at 18 carbons. The prior art does not disclose use of the high molecular weight wax esters commonly found in jojoba extract as a fungicidal agent.

Jojoba extract has been reported as a pretreatment to prevent powdery mildew infection on grape plants (Canadian Patent Application No. 2,103,014). Jojoba extract was previously thought to have limited commercial application. The mechanism of control was thought to be as a physical barrier similar to horticultural oils which have been known for many years. In our studies, it has been surprisingly determined that the wax esters found in jojoba extract are not merely an inert physical barrier but also a broad spectrum fungicide. This discovery gives rise to commercially important applications.

In modem agriculture and horticulture the avoidance of unnecessary environmental loading is a key commercial advantage. Both horticulture oils and fungicides are applied repeatedly during the early stages of the growing season to prevent the powdery mildew infection from becoming established. The oils and fungicides are applied multiple times per growth cycle to prevent powdery mildew infection and then again if powdery mildew appears. Since many crops have two or more growing cycles per year, this leads to yet more fungicidal treatments. As a result, the frequent fungicidal applications lead to a build up of that particular chemical agent in the environment (environmental loading). Conversely, application of the wax esters of the subject invention can be limited to instances where a powdery mildew infection occurs. Thus, wax ester fungicidal treatments are limited to a few, if any, application per growing season and environmental loading is significantly lowered.

Less fungicidal applications result in a lower cost to growers.

In addition, the wax ester fingicidal agent is rainfast after the application has dried. Thus, the wax ester fungicidal agent kills existing infections and then prevents further infection over time. Jojoba extract has the ability to eradicate fungicidal tolerant or resistant powdery mildew strains that have evolved under current fingicidal protocols. Therefore, use of a jojoba extract as a fungicide is economically efficient and significantly reduces environmental loading.

The wax esters provide a greater degree of safety than horticultural oils and fungicides. Many of the existing horticultural oils and fungicides are generally not environmentally safe in their application. For example, use of petroleum oils and sulfuric fingicidal applications for powdery mildew are restrained under the federal regulations as they have serious environmental ramifications if applied in a concentrated form or in high volume. Further, the fungicides and oils are generally volatile. Thus, use of these materials is hazardous to workers applying the chemical. Another popular fungicide, sterol inhibitors, are also heavily regulated because of their environmental impact and the residues they leave on edible crops. The wax esters used in this invention provide particular stability and are significantly less volatile than horticultural oils. Specifically, the greater degree of unsaturation and the long carbon chains, which are almost twice as long as fatty acid oils, enhances stability and non-volatility. Jojoba extract is a safe and natural application and is not heavily impacted by regulatory laws.

Additionally the wax esters of the subject invention have other advantages which make them a particularly effective as a fungicidal agent. For example, the long chained wax esters are generally non-toxic for predator, beneficials and honeybees. The wax ester agent is generally non-phytotoxic at the preferred range of use and, unlike many of the prior art fungicides mentioned above, never "bum" plant tissue. In fact the wax esters have been found to promote photosynthesis and stomatal conductance in the host plant.

SUMMARY OF THE INVENTION

It has been discovered that wax esters having high molecular weight are a particularly effective and safe fungicidal application for eradicating powdery mildew disease on plants. This invention provides a method for eradicating an existing powdery mildew fungus infection on plants with an emulsion of wax esters comprising wax esters of between 36 to 44 carbon atoms and a surfactant. The wax esters are derived from the esterification of monoethylenic acids and monoethylenic alcohols having between 18 and 24 carbons and are combined with a surfactant at a concentration of between 1% v/v and 10% v/v of the total wax esters composition.

Thus, in a preferred embodiment the invention provides a method of eradicating powdery mildew disease on a plant wherein the method comprises spraying an aqueous emulsion of a wax esters onto a plant in an amount sufficient to reduce powdery mildew infection in sprayed plants by at least 50% when compared to plants not sprayed with wax esters and wherein at least 50% v/v of the wax ester is comprised of carbon chains of 36 to 44 carbons atoms, with the proviso that spraying is conducted when i) the ambient temperature is below 80.degree. F. and is not conductive to white fly infestation and ii) the plant is not exhibiting anthesis.

In a preferred embodiment of the present invention, the wax esters are derived from the esterification of monoethylenic acids and monoethylenic alcohols and are combined with a suitable surfactant. The monoethylenic acids are chosen from a range of 18 to 22 length carbon chains and the monoethylenic alcohols from a range of 20 to 24 length carbon chains.

In another preferred embodiment of the present invention, 50% to 90% v/v of the wax esters are a mixture of eicosenyl eicosenoate and docosenyl eicosenoate and are combined with a suitable surfactant.

In yet another preferred embodiment of the present invention, the wax esters are comprised of a mixture of wax esters which are 85% similar in type and concentration to the wax ester found in naturally occurring jojoba extract and are combined with a suitable surfactant.

In yet another preferred embodiment of the present invention, the wax esters are jojoba extract and are combined with a suitable surfactant.

DEFINITIONS

The term "emulsion," as used herein refers to a stable mixture of two or more immiscible liquids held in liquid suspension. The mixture may be stabilized by the presence of emulsifiers or surfactants.

The term "aqueous emulsion," as used herein refers to preparations of a liquid wax or oil distributed in small globules throughout the body of a second liquid which is water. When the dispersed liquid is an oil or wax and is in the discontinuous phase and the dispersion medium is in the continuous phase it is an oil in water emulsion, whereas when water or aqueous solution is the dispersed phase and oil, wax or is the continuous phase, it is known as a water in oil emulsion..

The term "surfactants," as used herein refers to emulsifiers, detergents, surface active agents, anti-foaming agents or compounds which reduce surface tension when dissolved in water of a water solution, or which reduce interfacial tensions between two liquids. Thus the surfactant changes the properties of a solvent so that immiscible liquids may be more easily stabilized. Fundamentally, a surfactant is a single molecule comprised of two structurally dissimilar groups of opposing solubility tendencies, one which has an affinity for the phase and the other which is antipathic to the medium. The surfactant causes adsorption at the solution's interfaces, orientates the adsorped surfactant ions or molecules , promotes micelle formation in the bulk of the solution, and orientates the surfactant ions or molecules in the micelle, thereby increasing the solubility of the solvent and stabilizing the mixture.

The term "wax esters," as used herein refers to esters of long chain, even-numbered fatty acids and monohydric, straight chain, aliphatic alcohols, or sterols. Waxes are usually ester mixtures often accompanied by small percentages of free fatty acids or high molecular weight unbranched hydrocarbons. The wax acids and wax alcohols usually have a similar number of carbon atoms and are very hydrophobic.

The term "monoethylenic acids," as referred herein refers to carboxylic acid organic compounds where the carboxyl group is attached to one end of a hydrocarbon and the hydrocarbon contains a single double bond.

The term "monoethylenic alcohols," as referred herein refers to organic compounds where one or more hydroxyl groups (OH) are present in a hydrocarbon molecule with no more than one hydroxyl group attached to a single carbon atom and which also includes a single double bond in the hydrocarbon molecule.

The term "non-ionic surfactants," as referred to herein refers to surfactants, detergents or emulsifiers

which do not ionize in water and thus are not subject to hydrolysis by aqueous solutions of acid or alkali.

The term "siloxanes," as referred to herein refers to straight chain compounds consisting of silicon atoms single-bonded to oxygen and arranged so that each silicon atom is linked with four oxygen atoms. In some cases, hydrogen may replace two or more of the oxygens.

The term "polysiloxanes," as referred herein refers to siloxane chains wherein some of the oxygens are replaced with organic substituents so that a linear polymer results.

DETAILED DESCRIPTION

Introduction

Fungal infection in the agricultural industry represents a significant loss for growers in that fungal growth on crops may inhibit production of foliage, fruit or seeds, and lower the overall quality of a cultivated crop. Powdery mildew infection of crop plants represents about 25% of all fungal disease in agricultural and horticultural cultivation. Current treatments for eradicating powdery mildew have damaging environmental side effects, are often not particularly effective and can be damaging to the host plant itself.

It has recently been found that long chain wax esters have special utility as fungicidal agents. Particularly, aqueous emulsions of wax esters with a suitable surfactant, wherein the wax esters are the esterification product of monoethylenic acids of between 18 and 22 carbon chains and monoethylenic alcohols of between 20 to 24 carbon chains, are useful as fungicidal agents. This invention provides methods of use for wax esters as fungicidal agents for the eradication of existing powdery mildew infection on plants.

Wax Ester Sources

The wax esters of this invention are most conveniently extracted from Simmondsia chinensis (Jojoba). S. Chinensis is grown commercially for its wax esters. However, this invention is not intended to be limited by the origin of the wax esters of either synthetically or biologically origin. Sexual crosses between species is the genus. Related species in the genus are expected to yield novel plants which produce the wax esters for use in this invention.

Jojoba extract is more than 97% wax esters. Wax esters are derived from one molecule of a long-chain alcohol esterified with one long-chain fatty acid. Jojoba oil contains no glycerides, very little (1 percent) free acid or alcohol, and almost no hydrocarbons, steroids or other contaminants. Carbon chains of 18 and 24 atoms long make up about 93% of the acids and alcohols in the wax esters.

The unsaturated acid components of jojoba's wax esters are mostly a mixture of eicosanoic (C.sub.20), docosanoic (C.sub.22) and octadecanoic (C.sub.18) acids. The unsaturated alcohols are a mixture of eicosanol and docosanol, with smaller quantities of tetracosanol(C.sub.24) and alcohols of lower molecular weight. Over 85% of the esters present in jojoba oil are combinations of C.sub.20 and C.sub.22 acids and alcohols. The double bond position on the acids and alcohols typically falls between carbon 11 and carbon 12, and between carbon 13 and carbon 14.

More accurately, the alcohol content is comprised of 43.8% Eicos-1 -enol, 44.9 % Docos-13 enol and 8.9% Tetracos-15-enol (C.sub.24). The acid content consists of 71.3% Eicos-11-enoic acid, 13.6%

Docos-13-enoic acid and 10.1% Octdec-9-enoic acid. Percentage's of composition components at or below 2% are defined as trace components and are not included in this description.

Further, the wax esters present in jojoba oil typically break down to 30.9% Eicosenyl Eicosenoate (C.sub.40), 43.2% Docosenyl Eicosenoate (C.sub.42), 7.6% Eicosenyl Docosenoate (C.sub.42), 6.2% Tetracosenyl Eicosenoate (C44) and 5.9% Eicosenyl Octadecenoate (C.sub.38). Percentage's of components below 2% were defined as trace components and not included in the above description.

Extraction of Wax Esters From Natural Sources

The extraction of wax esters from jojoba can be carried out by any standard technique as is used in the industry for the extraction of fats or waxes from seeds, beans or nuts. For example, crushing or pressing the seeds and collecting the liquid wax is one such technique. Other techniques contemplated may be the use of solvents to extract the liquid wax. Solvents such as benzene, hexane, heptane and carbon tetrachloride have been shown to readily extract the desired wax esters in a satisfactory yield without special difficulty or affecting properties of the wax esters.

Synthesis of Wax Esters

Although it is preferable to extract the wax esters for the subject invention from naturally occurring sources such as jojoba plant, esters of this invention can be synthesized by a variety of standard esterification methods as is known in the art (see March, J., "Advanced Organic Chemistry-- Reactions, Mechanisms, and Structures", 4.sup.th ed., (1992)). For example, an acid catalyzed esterification of carboxylic acids with alcohols (the Fischer esterification reaction), wherein equilibrium is driven to the right, is one such esterification technique. Techniques commonly used to drive this reaction to the right include adding an excess of reactant (usually the alcohol), removal of the ester or water product by distillation, or removal of the water product by azeotropic distillation, use of a dehydrating agent or a molecular sieve. One skilled in the art would appreciate that this is just one of several esterification reactions available to the synthesize the wax esters contemplated in this invention.

As described above, the esterification reactions would be carried out with monoethylenic acid and alcohol precursors comprising carbon chains from about 18 to 24 carbon atoms, which are commonly sold by a variety of vendors. For the purposes of illustration, purchasing 13-docosaenoic acid and 11-eicosenol from Sigma-Aldrich Fine Chemicals Co. (see Sigma Catalog for Biochemicals and Reagents for Life Sciences, pg. 417 and 407 respectively, (1999)), combining these two precursors with an acid catalyst such as H.sub.2 SO.sub.4 or TsOH while drawing off the product ester or water by distillation would give the wax ester docosenol eicosenoate, a wax ester whose use as a fingicidal agent is detailed in this invention.

Application of Wax Ester Fungicidal Agents

The wax ester fungicidal agent may be applied to a wide variety of plants which are susceptible to powdery mildew infection. Such plant types would include bushes, grasses, vines, miniatures, trees, vegetable or fruit plants, ornamental plants or hybrids. For example, the subject wax ester fingicidal agent is particularly well suited for eradicating powdery mildew infections for apple trees, eggplant, tomatoes, strawberries, pea, squash and cucumber plants, to name but a few.

The wax ester fungicidal agent can be used to destroy various species of the fungus which cause powdery mildew disease. While generally a fungus species which causes powdery mildew disease is

in many cases specific to a single plant species, in other cases a powdery mildew fungus may infect a range of plants. Thus, the present wax ester fingicidal agent may be used to treat a wide variety of plants based upon their susceptibility to a powdery mildew fungus species. The following list, which is not meant to be limiting, details various species of powdery mildew causing fungus and the range of hosts which may be infected by the fungus species which are treatable by the subject invention. One skilled in the art will appreciate the vast number of plant species and varieties which exist and that efforts to breed new species and varieties that are constantly attempted. Thus, other species of plants which may be treated or species of powdery mildew fungus which may be destroyed by the subject invention would be obvious to any person skilled in the art.

Botanical name	Common name
ERYSIPHE CICHORACEARUM:	
Achillea	Yarrow
Ajuga	Carpet bugle
Alcea	Hollyhock
Antirrhinum	Snapdragon
Aster	
Baccharis	Coyote bush
Begonia	
Calendula	Pot marigold
Centaurea	Bachelor's button
Chrysanthemum	
Cineraria	
Citrullus	Watermelon
Cosmos	
Cotinus	Smoke tree
Dahlia	
Eucalyptus	
Gerbera	Transvaal daisy
Hebe	
Helianthus	Sunflower
Lactuca	Lettuce
Myosotis	Forget-me-not
Papaver	Poppy
Rununculus	
Rhus	Sumac
Rudbeckia	Black-eyed Susan
Salpiglossis	Painted tongue
Salvia	
Spiraea	
Verbena	
Zinnia	
ERYSIPHE POLYGONI:	
Amaranthus	Amaranth
Aguilegia	Columbine
Begonia	
Beta	Beet
Brassica	Cabbage family
Delphinium	California poppy
Eschscholzia	
Iberis	Candytuft
Lathyrus	Sweet pea
Lobularia	Sweet alyssum
Phoseolus	Beans
Pisum	Peas
Raphanus	Radish
Vinca	Periwinkle
Viola	Pansy, viola
MICROSHAERA ALNI:	
Alnus	Alder
Corylus	Hazelnut

Lonicera	Honeysuckle
Platanus	Plane tree
Quercus	Oak
Symphoricarpos	Snowberry
Syringa	Lilac
MICROSHAERA SPECIES:	
Acacia	
Catalpa	
Ceanothus	
Erica	Heath
Euonymus	
Hydrangea	
Juglans	Walnut
Lagerstroemia	Crapemyrtle
Ligustrum	Privet
Liriodendron	Tuliptree
Lonicera	Honeysuckle
Magnolia	
Passiflora	Passion flower
Platanus	Plane tree
Populus	Poplar
Raphiolepis	India hawthorn
Rhododendron	Rhododendron, azalea
Robinia	Locust
Vaccinium	Blueberry
Viburnum	
PHYLLATINIA CORYLEA:	
Aesculus	Horse chestnut
Cornus	Dogwood
Philadelphus	Mock orange
Quercus	Oak
Rubus	Brambles
PODOSPHAERA SPECIES:	
Acer	Maple
Fraxinus	Ash
Malus	Apple, crabapple
Photinia	
Prunus	Peach, plum,etc.
Pyracantha	Firethorn
Pyrus	Pear
Spiraea	
SPHAEROTHECA FULIGINEA:	
Cucumis	Cantaloupe
Cucumis	Cucumber
Curcubita	Winter squash
SPHAEROTHECA SPECIES:	
Cotoneaster	
Crataegus	Hawthorn
Dianthus	Sweet William, carnation, pink
Erica	Heath
Fragaria	Strawberry
Gaillardia	Blanket flower
Heuchera	Coral bells
Kalanchoe	
Nicotiana	
Petunia	
Phlox	
Potentilla	Cinquefoil
Ribas	Currant, gooseberry
Rosa	Rose
Tolmiea	

The wax ester fungicidal agent of this invention may be formulated into various forms such as solution, wettable powder, emulsion or spray, by mixing with any suitable solid or liquid carrier such as water. It is preferred that the wax ester application is applied in the form of an aqueous emulsion and such emulsions may also comprise a surfactant or combination of surfactants.

The wax ester agent may be applied by any of the methods typically known and used in the agricultural industry for the application of a chemical. Preferably, the wax ester fingicidal agent would be applied by any common spraying technique, including crop dusting by airplane or vehicle. However, the most preferable method for application of the subject wax ester agent is by any ground or hand sprayer which is commonly used in the agricultural industry.

Preferably, the wax ester emulsion is sprayed over the entire plant just to the point of runoff. Alternatively, specific points of infection may be treated directly without effecting other parts of the plant. That is, a single bunch of leaves or flowers on a plant may be sprayed with care given not to apply the wax ester emulsion over the whole of the plant.

The surfactants which can be employed in the wax ester fungicidal agent can be any of the non-phytotoxic surfactants which are customarily used in preparing agricultural formulations. The surfactant would be one which adequately increases the solubility of the wax ester in water and which stabilizes the mixture by increasing break time so that the wax ester emulsion stays in the emulsified state long enough to be applied over a large acreage. Further, a surfactant should be chosen which increase the spreadability of the wax ester fingicidal agent, so that the wax ester solution spreads at the same rate as the water phase over the various treated surface. In this way a filmy, uniform and rainfast application of the wax esters is achieved. Specifically, it is preferred for the present invention that the wax esters be mixed with a non-ionic surfactant, a siloxane or a polysiloxane. Surprisingly, it was found that the optimal composition was one where the wax esters were mixed with a non-ionic detergent to increase solution stability and then with a polysiloxane to enhance spreadability. Thus, the most preferred composition is one in which a combination of surfactants are employed. These type of surfactants and their use are well known in the art. A preferable non-ionizable surfactant would be IGEPAL CO [nonylphenoxypoly(ethyleneoxy)ethanol] or IGEPAL CA [octyphenoxypoly (ethyleneoxy)ethanol], manufactured by Rhone-Poulenc. Additionally, a preferable polysiloxane surfactants would be a polyether-polymethylsiloxane-copolymer such as Break-Thru.RTM. OE 441, a polysiloxane manufactured by Goldschmidt Chemical Corporation.

The wax ester fingicidal agent of the present invention can be diluted in order to facilitate its application in the field. Preferably, the wax ester/surfactant mixture would be diluted in water to form an aqueous emulsion for foliar application to plants. The dilution should be at a concentration which eradicates powdery mildew fungus without injuring the host plant. It is preferred that the wax ester/surfactant mixture be diluted from 0.25% v/v to about 5% v/v in water for optimum fungus eradication. For example, a 0.25% to 0.5% emulsion would be produced by mixing 1-2 pints of the wax ester/surfactant composition with approximately 40 to 50 gallons of water, for treatment of approximately one acre of crop land. One skilled in the art would appreciate that the stated volume of wax ester fungicide for treatment of one acre represents an application for intermediate foliage. The volume would be increased when the foliage of the treated plants is mature and decreased when the foliage is relatively new.

In addition to large scale applications for farms, greenhouse and agribusiness crop lands, the wax ester fingicidal agent can be employed as a fungal eradicator for home and garden plants. Thus, it is contemplated that the wax ester agent is also used in a ready-to-use formulation for home use. The

preferred concentrations of wax ester to surfactant to water is consistent as to what has been described herein. Preferably, the ready-to-use formulation would be 5% wax ester, 3.5% non-ionic surfactant and 3.5% siloxane or polysiloxane surfactant, all of which is diluted in water to form an 12% wax ester/surfactant concentration in water. In addition, a non-diluted wax ester/surfactant preparation of the same proportions above may be formulated as a refill product, where the refill product will later be diluted at the home by approximately 1 ounce of wax ester/surfactant to 1 quart of water.

Those skilled in the art will appreciate that the time of application of the wax ester fungicidal agent is determined by the particular characteristics and environment of the plant species to be treated. Generally, the wax ester fungicidal agent can be applied at any time in the growth cycle. Preferably, the agent is applied immediately when powdery mildew infection has been found on the host plant. In that way the infection may be eradicated before it spreads to other plants. Since the wax ester agent is rainfast, it will continue to prevent infection over an extended time. Further, the fungus causing powdery mildew will overwinter inside host plants. Therefore, where there has been prior infection in the previous season, the wax esters can be applied to the plant early in the next growing season, most preferably on growth tips or young plant tissue, to prevent reinfection as well as infection of nearby plants.

This invention represents a second horticultural use for jojoba extract. Previously jojoba extract was used for white fly control. White fly infestation is associated with high heat and moisture, in a range from 80.degree. F. to 100.degree. F., and most commonly between 85.degree. F. to 95.degree. F. In contrast, powdery mildew is a disease associated with relatively cool temperatures. Thus, the application of wax esters for control of powdery mildew is made when temperature is below 80.degree. F., and preferably between 55.degree. F. to 65.degree. F. when powdery mildew first begins to appear.

The wax esters of the subject invention exhibits excellent fruit thinning abilities. Therefore, application of the wax esters as a fingicidal agent is optimally made before or after anthesis. However, the subject wax ester fungicidal agent may be applied at the flowering stage of a plant but greater care would be taken to ensure that the wax esters are not applied to the fruiting body. However, if a powdery mildew infection exists at the time of harvest, application of the wax ester fungicidal agent can still effectively be made.

While a single application of the wax ester fungicidal agent should be satisfactory for effective fungus eradication, the wax ester fungicidal agent may be applied multiple times to achieve the desired effect. However, in applications wherein the wax ester agent make up more than 2.5% of the aqueous solution by volume, a subsequent application cannot be applied for at least 10 days have elapsed or host injury may occur. Further, applications at or above 5% wax ester emulsion may result in an phytoxic effect on the leaves.

EXAMPLES

The following example is offered to illustrate, but not to limit the claimed invention.

Example 1

Example 1 provides a preparation of a wax ester and surfactant composition diluted water to form an aqueous emulsion for use as a fungicidal agent. Specifically, herein is provided the preparation of a composition of wax esters which resemble in type and concentration the wax esters found in jojoba

extract by at least 85%, with a combination of surfactants to increase stability and spreadability of the wax ester solution, in an aqueous emulsion, for eradicating a powdery mildew infection on a plant.

A solution containing 93% v/v of a wax ester mixture comprising 43% docosenyl ecosenoate, 30% eicosenyl eicosenoate, 7.5% eicosenyl eicosenoate, 6% tetracosenyl eicosenoate and 5% eicosenyl octadecenoate, with a 7% mixture of surfactants was prepared. The solution was prepared by mixing together 93% jojoba extract v/v with 3 % v/v a non-ionic type surfactant and 4% polysiloxane type surfactant. The non-ionic surfactant was Igepal CA-520 from Rhone-Poulenc, which is an octylphenol ethoxylate containing 5 moles by weight of ethylene oxide. The polysiloxane used was Break-Thru.RTM. OE-441 from Goldschmidt Chemical Corporation, which is a polyether-polymethylsiloxane-copolymer. The wax ester and surfactant composition was mixed for 20 minutes by return flow agitation. The resultant solution contained the wax esters at the desired stability and spreadability to effectively destroy powdery mildew fungus infection over a plant surface. The wax ester solution can then be diluted in water to form an aqueous emulsion for application to a plant.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

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US PATENT & TRADEMARK OFFICE

PATENT FULL TEXT AND IMAGE DATABASE

United States Patent **6,069,299**
Broadway , et al. **May 30, 2000**

Fungus and insect control with chitinolytic enzymes

Abstract

The present invention relates to chitinolytic enzymes which have chitinolytic activity under alkaline conditions as well as DNA molecules encoding these enzymes and expression systems, host cells, and transgenic plants and plant seeds transformed with such DNA molecules. A chitinolytic enzyme can be applied to a plant or plant seed under conditions effective to control insects and/or fungi on the plant or plants produced from the plant seed. Alternatively, transgenic plants or transgenic plant seeds transformed with a DNA molecule encoding a chitinolytic enzyme can be provided and the transgenic plants or plants resulting from the transgenic plant seeds are grown under conditions effective to control insects and/or fungi.

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Appl. No.: **025691**
Filed: **February 18, 1998**

Current U.S. Class:	**800/279**; 435/69.1; 435/200; 435/252.3; 435/320.1; 435/418; 435/419; 435/468; 536/23.7; 800/288; 800/298; 800/302; 800/306; 800/307; 800/308; 800/309; 800/310; 800/312; 800/313; 800/314; 800/320; 800/321; 800/323
Intern'l Class:	C12N 005/04; C12N 015/31; C12N 015/56; C12N 015/82; A01H 005/00
Field of Search:	435/320.1,69.1,418,419,468,410,252.3,200 424/93.2 800/278,279,288,295,298,301,302,317,320,306,307-310,312-313,314,321,323 536/23.7

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Goverment Interests

The present invention was developed with support under USDA/NRI Grant No. 95-37302-1904. The U.S. Government may have certain rights.

Claims

What is claimed:

1. An isolated DNA molecule encoding a Streptomyces albidoflavus chitinolytic enzyme having chitinolytic activity under alkaline conditions.

2. An isolated DNA molecule according to claim 1, wherein the chitinolytic enzyme is selected from the group consisting of chitobiosidase and endochitinase.

3. An isolated DNA molecule according to claim 2, wherein the chitinolytic enzyme is chitobiosidase.

4. An isolated DNA molecule according to claim 3, wherein the chitobiosidase has a molecular mass of 34 kD and an isoelectric point of less than 3.0.

5. An isolated DNA molecule according to claim 3, wherein the chitobiosidase has an amino acid sequence comprising SEQ. ID. No. 1.

6. An isolated DNA molecule according to claim 2, wherein the chitinolytic enzyme is an endochitinase.

7. An isolated DNA molecule according to claim 6, wherein the endochitinase has a molecular mass of 45 kD and an isoelectric point of about 6.5.

8. An isolated DNA molecule according to claim 6, wherein the endochitinase has an amino acid sequence comprising SEQ. ID. No. 3.

9. An expression system transformed with the DNA molecule according to claim 1.

10. An expression system according to claim 9, wherein the chitinolytic enzyme is selected from the

group consisting of chitobiosidase, endochitinase, and combinations thereof.

11. An expression system according to claim 9, wherein the DNA molecule is in proper sense orientation and correct reading frame.

12. A host cell transformed with the DNA molecule according to claim 1.

13. A host cell according to claim 12, wherein the chitinolytic enzyme is selected from the group consisting of chitobiosidase, endochitinase, and combinations thereof.

14. A host cell according to claim 12, wherein the host cell is a bacterial cell.

15. A host cell according to claim 12, wherein the host cell is a plant cell.

16. A host cell according to claim 12, wherein the host cell contains an expression system transformed with the DNA molecule.

17. A transgenic plant transformed with the DNA molecule according to claim 1.

18. A transgenic plant according to claim 17, wherein the chitinolytic enzyme is selected from the group consisting of chitobiosidase, endochitinase, and combinations thereof.

19. A transgenic plant according to claim 18, wherein the chitinolytic enzyme is chitobiosidase.

20. A transgenic plant according to claim 18, wherein the chitinolytic enzyme is an endochitinase.

21. A transgenic plant according to claim 17, wherein the plant is selected from the group consisting of ***Gramineae,*** Liliaceae, Iridaceae, Orchidaceae, Salicaceae, Ranunculaceae, Magnoliaceae, Cruciferae, Rosaceae, Leguminosae. Malvaceae, Umbelliferae, Labitatae, Solanaceae, Cucurbitaceae, Compositae, and Rubiaceae.

22. A transgenic plant seed transformed with the DNA molecule according to claim 1.

23. A transgenic plant seed according to claim 22, wherein the chitinolytic enzyme is selected from the group consisting of chitobiosidase, endochitinase, and combinations thereof.

24. A transgenic plant seed according to claim 22, wherein the plant seed is selected from the group consisting of ***Gramineae,*** Liliaceae, Iridaceae, Orchidaceae, Salicaceae, Ranunculaceae, Magnoliaceae, Cruciferae, Rosaceae, Leguminosae, Malvaceae, Umbelliferae, Labitatae, Solanaceae, Cucurbitaceae, Compositae, and Rubiaceae.

25. A method of insect control for plants comprising:

providing a transgenic plant or plant seeds transformed with a DNA molecule according to claim 1 and

growing the transgenic plant or transgenic plants produced from the transgenic plant seeds under conditions effective to control insects.

26. A method according to claim 25, wherein a transgenic plant is provided.

27. A method according to claim 25, wherein a transgenic plant seed is provided.

28. An isolated DNA molecule encoding a chitinolytic enzyme having chitinolytic activity under alkaline conditions, wherein said DNA molecule has a nucleotide sequence of SEQ. ID. Nos. 2 or 4 or a nucleotide sequence which hybridizes to the nucleotide sequence of SEQ. ID. Nos. 2 or 4 under stringent conditions.

29. An isolated DNA molecule according to claim 28, wherein the DNA molecule has a nucleotide sequence which hybridizes to the nucleotide sequence of SEQ. ID. Nos. 2 or 4 under stringent conditions.

30. An isolated DNA molecule according to claim 29, wherein the stringent conditions are 65.degree. C. for 20 hours in a medium containing 1M NaCl, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.1% sodium dodecyl sulfate, 0.2% ficoll 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 50 .mu.m g/ml E. coli DNA.

31. An expression system transformed with the DNA molecule according to claim 28.

32. A host cell transformed with the DNA molecule according to claim 28.

33. A host cell according to claim 32, wherein the host cell is a bacterial cell.

34. A host cell according to claim 32, wherein the host cell is a plant cell.

35. A host cell according to claim 32, wherein the host cell contains an expression system transformed with the DNA molecule.

36. A transgenic plant transformed with the DNA molecule according to claim 28.

37. A transgenic plant according to claim 35, wherein the plant is selected from the group consisting of ***Gramineae,*** Liliaceae, Iridaceae, Orchidaceae, Salicaceae, Ranunculaceae, Magnoliaceae, Cruciferae, Rosaceae, Leguminosae, Malvaceae, Umbelliferae, Labitatae, Solanaceae, Cucurbitaceae, Compositae, and Rubiaceae.

38. A transgenic plant seed transformed with the DNA molecule according to claim 28.

39. A transgenic plant seed according to claim 38, wherein the plant seed is selected from the group consisting of ***Gramineae,*** Liliaceae, Iridaceae, Orchidaceae, Salicaceae, Ranunculaceae, Magnoliaceae, Cruciferae, Rosaceae, Leguminosae, Malvaceae. Umbelliferae, Labitatae, Solanaceae, Cucurbitaceae, Compositae, and Rubiaceae.

40. A method of insect control for plants comprising:

providing a transgenic plant or plant seeds transformed with a DNA molecule according to claim 28 and

growing the transgenic plant or transgenic plants produced from the transgenic plant seeds under conditions effective to control insects.

41. A method according to claim 40, wherein a transgenic plant is provided.

42. A method according to claim 40, wherein a transgenic plant seed is provided.

43. A DNA molecule according to claim 1, wherein the chitinoltic enzyme has chitinolytic activity only under alkaline conditions.

44. A DNA molecule according to claim 28, wherein the chitinolytic enzyme has chitinolytic activity only under alkaline conditions.

45. An isolated DNA molecule according to claim 29, wherein the DNA molecule has a nucleotide sequence which hybridizes to the nucleotide sequence of SEQ. ID. No. 2 under stringent conditions.

46. An isolated DNA molecule according to claim 29, wherein the DNA molecule has a nucleotide sequence which hybridizes to the nucleotide sequence of SEQ. ID. No. 4 under stringent conditions.

47. An isolated DNA molecule according to claim 28, wherein the DNA molecule has a nucleotide sequence of SEQ. ID. No. 2.

48. An isolated DNA molecule according to claim 28, wherein the DNA molecule has a nucleotide sequence of SEQ. ID. No. 4.

Description

FIELD OF THE INVENTION

The present invention relates to chitinolytic enzymes which are active against insects under alkaline conditions.

BACKGROUND OF THE INVENTION

The introduction of synthetic organic pesticides following World War II brought inestimable benefits to humanity and agricultural economic profitability. Application of broad-spectrum pesticides is the primary method used for controlling fungal and insect pests. For example, the widescale deployment of DDT resulted in the complete riddance, from entire countries, of serious public pests such as malaria mosquitoes. However, there were warnings about the hazard of unilateral approaches to pest control.

The development of new pesticides and the increasing amounts of pesticides used for pest control are closely correlated with the development of pest resistance to chemicals. The number of pesticide resistant species has greatly increased since the adoption of DDT in 1948. As a result, by the 1980s, the number of reports of pesticide resistance for arthropod pests was listed as 281, for plant pathogens 67, and for weeds 17. These numbers have steadily increased to the present day. Thus, the need for biological control agents, especially those with broadbase activity is especially important.

One approach that is gaining significant attention is the use of agricultural cultivars that are resistant to pests. These cultivars can be developed by the transgenic introduction of target specific natural resistance factors. However, to enhance host-plant resistance, it is necessary first to identify and to characterize target-specific factors that will significantly reduce the population(s) of herbivorous

insect(s).

Only a limited number of natural products have been characterized and identified as effective defensive agents against herbivorous insects, few of these are proteins (e.g., proteinase inhibitors, arcelin, alpha-amylase inhibitors, lectins, endotoxin from Bacillus thuringiensis, and lipoxygenases), and even fewer are target specific (Duffey, et al., "Plant Enzymes in Resistance to Insects," In J. R. Whitaker and P. E. Sonnet (eds.), Biocatalysis in Agricultural Biotechnology, American Chemical Society, Washington, D.C. (1989); Gill, et al., "The Mode of Action of Bacillus thuringiensis Endotoxins," Ann. Rev. Entomol., 37:615-36 (1992); Hedin, P. A., "Plant Resistance to Insects," American Chemical Society, Washington, D.C., p. 375 (1983); Rosenthal, et al., "Herbivores--Their Interaction with Secondary Plant Metabolites," Academic Press, New York, p. 718 (1979). Identification and characterization of proteins as resistance factor(s) enables the isolation of gene(s) that encode(s) these proteins. These genes can be transgenically inserted into agricultural crops, which may enhance the resistance of these crops against herbivorous insects without altering desirable characteristics of the cultivar(s) (Fraley, et al., "Genetic Improvements of Agriculturally Important Crops," Cold Spring Harbor Laboratory, p. 120 (1988); Hilder, et al., "A Novel Mechanism of Insect Resistance Engineered into Tobacco," Nature, 330:160-63 (1987); Ryan, C. A., "Proteinase Inhibitor Gene Families: Strategies for Transformation to Improve Plant Defenses Against Herbivores," BioEssays, 10:20-24 (1989); Vaeck, et al., "Transgenic Plants Protected from Insect Attack," Nature, 328:33-27 (1987).

One target that has been selected is a structural polymer, chitin, which is present in insects and some fungi that attack plants, but is absent in higher plants and vertebrates. U.S. Pat. No. 4,751,081 follows this approach and is directed to novel chitinase producing bacteria strains for use in inhibiting chitinase sensitive plant pathogens (i.e. fungi and nematodes). However, the approach of U.S. Pat. No. 4,751,081 lacks flexibility.

The present invention is directed to controlling fungi and insects that attack plants.

SUMMARY OF THE INVENTION

The present invention relates to isolated DNA molecules encoding chitinolytic enzymes, which have chitinolytic activity under alkaline conditions, as well as to vectors, host cells, and transgenic plants and plant seeds transformed with these DNA molecules.

Another aspect of the present invention relates to a method of insect and/or fungus control for plants. This method involves applying the chitinolytic enzymes to plants or plant seeds under conditions effective to control insects and/or fungi on the plants or plants grown from the plant seeds.

As an alternative to applying the chitinolytic enzymes to plants or plant seeds in order to control insects and/or fungi on plants or plants grown from the seeds, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a chitinolytic enzyme and growing the plant under conditions effective to control insects and/or fungi. Alternatively, a transgenic plant seed transformed with a DNA molecule encoding the chitinolytic enzyme can be provided and planted in soil. A plant is then propagated from the planted seed under conditions effective to control insects and/or fungi.

The present invention is directed to effecting any form of insect and/or fungus control for plants.

For example, insect control or fungus control according to the present invention encompasses

preventing insects or fungi from contacting plants to which the chitinolytic enzymes have been applied, preventing direct damage to plants by feeding injury, causing insects or fungi to depart from such plants, killing insects or fungi proximate to such plants, interfering with feeding on plants by insects or fungi, preventing insects or fungi from colonizing host plants, preventing colonizing insects or fungi from releasing phytotoxins, etc. The present invention also prevents subsequent disease damage to plants resulting from insect or fungal invasion.

As a result, the present invention provides significant economic benefit to growers.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A to C show a time course of chitinolytic enzyme activity in culture filtrate from Streptomyces albidoflavus NRRL B-16746 and NRRL 21918. The liquid medium contained 0.5% chitin, while the glucose component was 0% (solid square), 0.01% (open triangle), 0.1% (open circle), 0.25% (solid triangle), and 0.5% (open square). FIG. 1A indicates endochitinase activity (units/ml), FIG. 1B indicates chitobiosidase activity (% nkatals), and FIG. 1C indicates glucosaminidase activity (% nkatals) at pH 9. These are representative data; the experiment was replicated twice with similar results.

FIGS. 2A-B show the polyacrylamide gel electrophoresis of dialyzed culture filtrate from Streptomyces albidoflavus NRRL B-16746. Lanes 1-8 contain samples collected after 0, 24, 48, 72, 96, 120, 144, 168, or 192 hrs. of culture, respectively. FIG. 2A shows native gel with Coomassie stain, while FIG. 2B shows native gel with overlay of fluorogenic substrate for endochitinases and chitobiosidases. C indicates the chitobiosidase band, G indicates the glucosaminidase band, and E indicates the endochitinase bands. These are representative data; the experiment was replicated 2 times with similar results.

FIG. 3 shows the influence of pH on chitinolytic activity in the culture medium from Streptomyces albidoflavus NRRL B-16746. Glucosaminidase activity is indicated by the open circles, chitobiosidase activity is indicated by open triangles, and endochitinase activity is indicated by the solid squares.

FIG. 4 shows the low pressure anion exchange chromatography of chitinolytic enzymes from Streptomyces albidoflavus NRRL B-16746. The solid line indicates total protein (optical density, 280 nm), the dashed line indicates chitobiosidase activity (% nkatals), and the dotted line indicates endochitinase activity (units/ml). These are representative data; the analysis was replicated more than 10 times.

FIGS. 5A-B show the polyacrylamide gel electrophoresis of chitinolytic enzymes from anion exchange chromatography. Lanes 1 and 2 contain endochitinases (peaks III and IV, respectively, from FIG. 8), and lane 3 contains chitobiosidases (peak II from FIG. 4). The gels were stained with Coomassie (FIG. 5A), or a chitinase-specific fluorogenic substrate (FIG. 5B). These are representative data; the determination of the number of chitinolytic enzymes was replicated more than 10 times.

FIGS. 6A-B show the isoelectric focusing gel of chitinolytic enzymes from anion exchange chromatography. The gels were stained with Coomassie (FIG. 6A), or a chitinase-specific fluorogenic substrate (FIG. 6B). For FIG. 6A, lane 1 contains endochitinases (peaks III from FIG. 8), lane 2 contains isoelectric focusing markers, lane 3 contains endochitinases (peaks IV from FIG. 8), and lane 4 contains chitobiosidases (peak II from FIG. 4). For FIG. 6B, lane 1 contains endochitinases

(peaks IV from FIG. 8), lane 2 contains endochitinases (peaks III from FIG. 8), and lane 3 contains chitobiosidases (peak II from FIG. 4). The isoelectric points of the chitobiosidases were <3.0, while the isoelectric points of the endochitinases were 6.4, 5.8-5.9, 5.7, 5.3, 5.1, and 5.0. These are representative data; the isoelectric point determinations were replicated more than 10 times.

FIG. 7 shows the SDS-polyacrylamide gel electrophoresis of chitinolytic enzymes from anion exchange chromatography. Lane 1 contains endochitinases (peak III from FIG. 8), lane 2 contains molecular weight markers, lane 3 contains endochitinases (peak IV from FIG. 8), lane 4 contains chitobiosidases (peak II, FIG. 4). The gel was stained with Coomassie. The proteins with chitobiosidase activity have molecular mass of 59, 45, 38.5, 27, and 25.5 kD. These are representative data; the molecular weight determinations were replicated more than 10 times.

FIG. 8 shows the perfusion anion exchange chromatography of endochitinases (FIG. 4, peak I) from Streptomyces albidoflavus NRRL B-16746. The solid line indicates total protein (optical density, 280 nm), the dashed line indicates sodium chloride gradient. Endochitinase activity occurred in peaks III and IV. These are representative data; the chromatographic separation was replicated 6 times.

FIG. 9 shows the strong anion exchange perfusion chromatography of semipurified chitinolytic enzymes. Total chitinolytic activity, as measured by hydrolysis of p-nitrophenyl .beta.-D-N,N'-diacetylchitobiose, was 0.14 nkat/peak I, 1.52 nkat/peak II, 0.26 nkat/peak III, <0.01 nkat/peak IV, 2.45 nkat/peak V, and 0.09 nkat/peak VI.

FIGS. 10A-B show polyacrylamide gel electrophoresis of the peaks collected from anion exchange perfusion chromatography (FIG. 9). FIG. 10A shows Coomassie stain to detect all proteins. FIG. 10B shows fluorogenic overlay to locate enzymes with chitinolytic activity. Based on previous experiments (Broadway, et al., "Partial Characterization of Chitinolytic Enzymes from Streptomyces albidoflavus," Lett. Appl. Microbiol., 20:271-76 (1995), which is hereby incorporated by reference), the upper (most acidic) two bands were chitobiosidases, while the lower (most alkaline) five bands were endochitinases.

FIG. 11 shows the development of Trichoplusia ni larvae on artificial diet supplemented with semipurified chitinolytic enzymes. Solid bars indicate larval weight as a percent of the mean weight of untreated controls. Stippled bars indicate the percent larvae that pupated. Hatched bars indicate the percent pupae that molted to adults. Vertical lines indicate .+-.1 SEM. Columns associated with an insect growth stage having similar letters are not significantly different (LSD test).

FIG. 12 shows the effect of dietary chitinolytic enzymes on survival of Myzus persicae, Bemisia argentifolii, and Hypothenemus hampei. For B. argentifolii, solid bars indicate % mortality after 18 h exposure, stippled bars indicate % mortality after 42 h exposure. For M. persicae, solid bars indicate % mortality after 24 h exposure, while stippled bars indicate % mortality after 48 h exposure. For H. hampei, solid bars indicate % mortality after 30d exposure. Vertical lines indicate .+-.1 SEM. Columns associated with a single time of observation and having similar letters are not significantly different (LSD test).

FIG. 13 shows the effect of dietary endochitinases or chitobiosidases on survival of Bemisia argentifolii. Endochitinase treatments I and II contained 10% sucrose and 0.5% peak I or II, respectively, from the anion exchange perfusion chromatography (FIG. 9), while chitobiosidase treatments IV and V contained 10% sucrose and 0.5% peak IV or V, respectively, from the anion exchange column (FIG. 9). Two controls were included: treatment VI contained 10% sucrose and 0.5% peak VI (no chitinolytic activity), and treatment C, which contained 10% sucrose. Vertical lines

indicate .+-.1 SEM. Columns with similar letters are not significantly different (LSD test).

FIG. 14 shows the biological activity of chitobiosidases against Botrytis cinerea and Fusarium oxysporum. Square indicates % inhibition of spore germination, triangle indicates % inhibition of germ tube elongation, dashed line indicates Botrytis cinerea, and solid line indicates Fusarium oxysporum.

DETAILED DESCRIPTION OF THE INVENTION

Chitin, an insoluble linear .beta.-1,4-linked polymer of N-acetyl-.beta.-D-glucosamine, is a structural polysaccharide that is present in all arthropods, yeast, most fungi, and some stages of nematodes. Chitinolytic enzymes are proteins that catalyze the hydrolysis of chitin by cleaving the bond between the C1 and C4 of two consecutive N-acetylglucosamines. There are three types of chitinolytic enzyme activity: (1) N-acetyl-.beta.-glucosaminidase (i.e., EC 3.2.1.30, abbreviated glucosaminidase), which cleaves monomeric units from the terminal end of chitin, (2) 1,4-.beta.-chitobiosidase (i.e., abbreviated chitobiosidase), which cleaves dimeric units from the terminal end of chitin, and (3) endochitinase (EC 3.2.1.14), which randomly cleaves the chitin molecule internally (Sahai, et al., "Chitinases of Fungi and Plants: Their Involvement in Morphogenesis and Host-Parasite Interaction," FEMS Microbiol. Rev., 11:317-38 (1993), which is hereby incorporated by reference). Two or three types of enzymes are often synthesized by a single organism (Harman, et al., "Chitinolytic Enzymes of Trichoderma harzianum: Purification of Chitobiase and Endochitinase," Phytopathology, 83:313-18 (1993), Neugebauer, et al., "Chitinolytic Properties of Streptomyces lividans," Arch. Microbiol, 156:192-97 (1991), Romaguera, et al., "Protoplast Formation by a Mycolase from Streptomyces olivaceoviridis and Purification of Chitinases," Enzyme Microb. Technol., 15:412-17 (1993), which are hereby incorporated by reference), which may enhance the speed and/or efficiency of degradation of chitin.

The chitinolytic enzymes of the present invention are particularly effective in controlling insects, because they are active under alkaline conditions. As a result, these enzymes can be ingested by insects and then attack the insects by degrading their chitin-containing, alkaline digestive tracts.

The present invention relates to chitinolytic enzymes which are active under alkaline conditions (i.e. at a pH greater than 7) alone but may also be active under neutral and/or acid conditions. The present invention also encompasses the DNA molecules encoding these enzymes. Examples of such chitinolytic enzymes are the following enzymes isolated from Streptomyces albidoflavus which have either chitobiosidase or endochitinase activity.

The chitobiosidase isolated from Streptomyces albidoflavus has an amino acid sequence of SEQ. ID. No. 1 as follows: Ala Pro Ala Ala Val Pro Ala His Ala Val Thr Gly Tyr Trp Gln Asn 1 5 10 15 - Phe Asn Asn Gly Ala Thr Val Gln Thr Leu Ala Asp Val Pro Asp Ala 20 25 30 - Tyr Asp Ile Ile Ala Val Ser Phe Ala Asp Ala Thr Ala Asn Ala Gly 35 40 45 - Glu Ile Thr Phe Thr Leu Asp Ser Val Gly Leu Gly Gly Tyr Thr Asp 50 55 60 - Glu Gln Phe Arg Ala Asp Leu Ala Ala Lys Gln Ala Asp Gly Lys Ser 65 70 75 80 - Val Ile Ile Ser Val Gly Gly Glu Lys Gly Ala Val Ala Val Asn Asp 85 90 95 - Ser Ala Ser Ala Gln Arg Phe Ala Asp Ser Thr Tyr Ala Leu Met Glu 100 105 110 - Glu Tyr Gly Phe Asp Gly Val Asp Ile Asp Leu Glu Asn Gly Leu Asn 115 120 125 - Ser Thr Tyr Met Thr Glu Ala Leu Thr Lys Leu His Glu Lys Ala Gly 130 135 140 - Asp Gly Leu Val Leu Thr Met Ala Pro Gln Thr Ile Asp Met Gln Ser 145 150 155 160 - Pro Glu Asn Glu Tyr Phe Lys Thr Ala Leu Val Thr Lys Asp Phe Leu 165 170 175 - Thr Ala Val Asn Met Gln Tyr Tyr Asn Ser Gly Ser Met Leu Gly Cys 180 185 190 - Asp Gly Gln Val Tyr Ala Gln Gly Thr Val Asp Phe Leu Thr Ala Leu 195 200 205 - Ala Cys Ile Gln Leu Glu Asn Gly Leu Asp Ala Ser Gln Val Gly Ile 210 215 220 - Gly Val Pro Ala Ser Pro Lys Ala Ala

Gly Gly Gly Tyr Val Glu Pro 225 230 235 240 - Ser Val Val Asn Asp Ala Leu Asp Cys Leu Thr Arg Gly Thr Gly Cys 245 250 255 - Gly Ser Phe Lys Pro Glu Lys Thr Tyr Pro Ala Leu Arg Gly Ala Met 260 265 270 - Thr Trp Ser Thr Asn Trp Asp Ala Asp Thr Gly Asn Ala Trp Ser Asn 275 280 285 - Val Val Gly Pro His Val Asp Asp Leu Pro 290 295

The chitobiosidase has a molecular mass of 34 kD and an isoelectric point of less than 3.0.

The chitobiosidase isolated from Streptomyces albidoflavus having an amino acid sequence of SEQ. ID. No. 1 is encoded by a DNA molecule having a nucleotide sequence of SEQ. ID. No. 2 as follows: GCGGCCGCTC CGGGCGGACG ACCGTACGGA CTCCTCGGCC GACCCCTGCG GGAACCCTTG 60 - ACAACCCCAT TGGTCTGGAC CAGTTTGGTG CCCATCGCGG TGGCCACCG T GCGCCAACTC 120 - CCCGCCCCCT CCCGGGTGGC GGGCCCCGTC GGCGCGTCCC CCCACGTCCG TGACTCCCCC 180 - CACCGGAGGC AGCAGTGGTA CGCACCTACC CCCTTCCGCA CCCCGGCCGG CGCCCCTCCA 240 - CGCCCGGCCT CCACCGCAGG GGCCGGCTGA CCGCCGCCCT CACCGCGGCC GTCCTCGGCG 300 - CCTCCGGGCT CGCCCTCACC GGCCCCGCGA CCGCCGGCGA GGGGGCCCCC GCCGCCCAGG 360 - CCGCCCCGGC CGCCGTACCG GCCCACGCGG TGACCGGTTA CTGGCAGAAC TTCAACAACG 420 - GCGCGACCGT GCAGACCCTC GCCGACGTGC CGGACGCCTA CGACATCATC GCCGTCTCCT 480 - TCGCCGACGC CACGGCCAAC GCGGGCGAGA TCACCTTCAC CCTCGACTCG GTCGGGCTCG 540 - GCGGCTACAC CGACGAGCAG TTCCGCGCCG ACCTCGCCGC CAAGCAGGCC GACGGCAAGT 600 - CGGTGATCAT CTCGGTCGGC GGCGAGAAGG GCGCGGTCGC CGTCAACGAC AGCGCCTCCG 660 - CCCAGCGCTT CGCCGACAGC ACCTACGCGC TGATGGAGGA GTACGGCTTC GACGGCGTCG 720 - ACATCGACCT GGAGAACGGC CTCAACTCCA CCTACATGAC CGAGGCCCTC ACCAAGCTCC 780 - ACGAGAAGGC CGGGGACGGC CTGGTCCTCA CCATGGCGCC GCAGACCATC GACATGCAGT 840 - CGCCCGAGAA CGAGTACTTC AAGACGGCGC TGGTCACGAA AGACTTCCTG ACCGCCGTCA 900 - ACATGCAGTA CTACAACAGC GGCTCGATGC TCGGCTGCGA CGGCCAGGTC TACGCGCAGG 960 - GCACCGTCGA CTTCCTCACC GCGCTCGCCT GCATCCAGCT GGAGAACGGT CTCGACGCCT1020 - CCCAGGTCGG CATCGGTGTC CCCGCCTCCC CGAAGGCGGC CGGCGGCGGC TACGTCGAGC1080 - CCTCCGTGGT CAACGACGCG CTGGACTGCC TGACCCGGGG CACCGGTTGT GGCTCGTTCA1140 - AGCCGGAGAA GACCTACCCG GCGCTGCGTG GCGCCATGAC CTGGTCGACC AACTGGGACG1200 - CCGACACCGG CAACGCCTGG TCGAACGTGG TCGGCCCGCA CGTCGACGAC CTGCCGTAAC1260 - CCCGGAGCCG GGCACCCGTC CGCTTCCCCC GCAC1294

The endochitinase isolated from Streptomyces albidoflavus has an amino acid sequence of SEQ. ID. No. 3 as follows: Gly Pro Gly Pro Gly Pro Arg Glu Lys Ile Asn Leu Gly Tyr Phe Thr 1 5 10 15 - Glu Trp Gly Val Tyr Gly Arg Asn Tyr His Val Lys Asn Leu Val Thr 20 25 30 - Ser Gly Ser Ala Glu Lys Ile Thr His Ile Asn Tyr Ser Phe Gly Asn 35 40 45 - Val Gln Gly Gly Lys Cys Thr Ile Gly Asp Ser Phe Ala Ala Tyr Asp 50 55 60 - Lys Ala Tyr Thr Ala Ala Glu Ser Val Asp Gly Val Ala Asp Thr Trp 65 70 75 80 - Asp Gln Pro Leu Arg Gly Asn Phe Asn Gln Leu Arg Lys Leu Lys Ala 85 90 95 - Lys Tyr Pro His Ile Lys Val Leu Trp Ser Phe Gly Gly Trp Thr Trp 100 105 110 - Ser Gly Gly Phe Thr Asp Ala Val Lys Asn Pro Ala Ala Phe Ala Lys 115 120 125 - Ser Cys His Asp Leu Val Glu Asp Pro Arg Trp Ala Asp Val Phe Asp 130 135 140 - Gly Ile Asp Leu Asp Trp Glu Tyr Pro Asn Ala Cys Gly Leu Ser Cys 145 150 155 160 - Asp Ser Ser Gly Pro Ala Ala Leu Lys Asn Met Val Gln Ala Met Arg 165 170 175 - Ala Gln Phe Gly Thr Asp Leu Val Thr Ala Ala Ile Thr Ala Asp Ala 180 185 190 - Ser Ser Gly Gly Lys Leu Asp Ala Ala Asp Tyr Ala Gly Ala Ala Gln 195 200 205 - Tyr Phe Asp Trp Tyr Asn Val Met Thr Tyr Asp Phe Phe Gly Ala Trp 210 215 220 - Asp Lys Thr Gly Pro Thr Ala Pro His Ser Ala Leu Asn Ser Tyr Ser 225 230 235 240 - Gly Ile Pro Lys Ala Asp Phe His Ser Ala Ala Ala Ile Ala

Lys Leu 245 250 255 - Lys Ala Lys Gly Val Pro Ala Ser Lys Leu Leu Leu Gly Ile Gly Phe 260 265 270 - Tyr Gly Arg Gly Trp Thr Gly Val Thr Gln Asp Ala Pro Gly Gly Thr 275 280 285 - Ala Thr Gly Pro Ala Thr Gly Thr Tyr Glu Ala Gly Ile Glu Asp Tyr 290 295 300 - Lys Val Leu Lys Asn Thr Cys Pro Ala Thr Gly Thr Val Gly Gly Thr 305 310 315 320 - Ala Tyr Ala Lys Cys Gly Ser Asn Trp Trp Ser Tyr Asp Thr Pro Ala 325 330 335 - Thr Ile Lys Thr Lys Met Thr Trp Ala Lys Asp Gln Gly Leu Gly Gly 340 345 350 - Ala Phe Phe Trp Glu Phe Ser Gly Asp Thr Ala Gly Gly Glu Leu Val 355 360 365 - Ser Ala Met Asp Ser Gly Leu Arg 370 375

The endochitinase has a molecular mass of 45 kD and an isoelectric point of about 6.5.

The endochitinase isolated from Streptomyces albidoflavus having an amino acid sequence of SEQ. ID. No. 3 is encoded by a DNA molecule having a nucleotide sequence of SEQ. ID. No. 4 as follows: GTCGACTGGT ACAACGTGAT GACCTACGAC TACTTCGGCA CCTGGGCCGC CCAGGGCCCG 60 - ACGGCGCCCC ACTCGCCGCT CACCGCCTAC CCGGGCATCC AGGGCGAGC A CAACACCTCC 120 - TCGGCCACCA TCGCCAAGCT GCGGGGCAAG GGCATCCCGG CGAAGAAGCT GCTGCTGGGC 180 - ATCGGCGCCT ACGGCCGCGG CTGGACCGGC GTCACCCAGG ACGCCCCCGG CGGCACCGCC 240 - ACCGGCCCGG CCGCCGGCAC CTACGAGGCG GGCAACGAGG AGTACCGGGT GCTGGCCGAG 300 - AAGTGCCCGG CCACCGGCAC CGCCGGCGGC ACCGCGTACG CCAAGTGCGG CGACGACTGG 360 - TGGAGTTACG ACACCCCTGA GACGGTGACG GGCAAGATGG CCTGGGCGAA GAAGCAGAAG 420 - CTCGGCGGTG CCTTCCTCTG GGAGTTCGCC GGCGACGGCG CCAAGGGCGA TCTGTTCAGG 480 - GCGATGCACG AGGGGCTGCG CTGACCGGCC GGGCACTCAC CCGGAACTGA CCCTTCCCGC 540 - ACGGCCGTCC GCCGTGGCAC CGGAGCTCCG GTCGCCGCGG CGGGCGGCCG TGTCCGCATG 600 - TCGCCACCCC CGCGCACCAG GCGCGATCCG GCCGAACTTT CCTTTGGTCC AGACCTCTTG 660 - ACCTCTGGTC CAGACCTTTT CTACTCTCGC CCCACTGCGG TGGGCACATC GGTCGTCGGT 720 - GCTCACGGGC GTCGCAGGGT TCCGCCCCCA TACGTCCGGA CCTCTTGAGG AGTACGCCTT 780 - GAGTACGGTT TCCCCCAGCA CCGACGGCGC CCGCAGCCGT CCCAGACCCC TCAGCCGCTT 840 - CCGCCGGCGC GCGCTGGCCG CGCTCGTCGG CCTCGCGGTC CCCTTCGCCG GGATGGTCGG 900 - CCTCGCCGCC CCCACCCAGG CCGCCGAGGC CGCGGCCGAC CCCAGCGCCT CCTACACCAG 960 - GACGCAGGAC TGGGGCAGCG GCTTCGAGGG CAAGTGGACG GTGAAGAACA CCGGCACCGC1020 - CCCCCTCAGC GGCTGGACCC TGGAGTGGGA CTTCCCCGCC GGAACCAAGG TGACCTCGGC1080 - CTGGGACGCC GACGTCACCA ACAACGGCGA CCACTGGACC GCCAAGAACA AGAGCTGGGC1140 - GGGGAGCCTC GCCCCCGGCG CCTCGGTCAG CTTCGGCTTC AACGGCACCG GCCCCGGCAC1200 - CCCCTCGGGC TGCAAGCTCA ACGGCGCCTC CTGCGACGGC GGCAGCGTCC CCGGCGACAC1260 - CCCGCCCACC GCCCCCGGCA CCCCCACCGC CAGTGACCTC ACCAAGAACT CGGTGAAGCT1320 - CTCCTGGAAG GCGGCCACCG ACGACAAGGG CGTCAAGAAC TACGACGTCC TGCGCGACGG1380 - CGCCAAGGTC GCCACCGTCA CCGCCACCAC CTTCACCGAC CAGAACCTCG CCCCCGGCAC1440 - CGACTACTCC TACTCGGTCC AGGCCCGCGA CACCGCCGAC CAGACCGGCC CGGTCAGCGC1500 - CCCCGTCAAG GTCACCACCC CCGGCGACGG CACGGGCCCC GGCCCCGGCC CCCGCGAGAA1560 - GATCAACCTC GGCTACTTCA CCGAGTGGGG CGTCTACGGC CGCAACTACC ACGTCAAAAA1620 - CCTGGTGACC TCCGGCTCCG CCGAGAAGAT CACCCACATC AACTACTCCT TCGGCAACGT1680 - CCAGGGCGGC AAGTGCACCA TCGGTGACAG CTTCGCCGCC TACGACAAGG CGTACACCGC1740 - CGCCGAGTCG GTCGACGGCG TCGCCGACAC CTGGGACCAG CCGCTGCGCG GCAACTTCAA1800 - CCAGCTCCGC AAGCTCAAGG CCAAGTACCC GCACATCAAG GTCCTCTGGT CCTTCGGCGG1860 - CTGGACCTGG TCCGGCGGCT TCACCGACGC CGTGAAGAAC

CCGGCCGCCT TCGCCAAGTC1920 - CTGCCACGAC CTGGTCGAGG ACCCGCGCTG GGCCGACGTC TTCGACGGCA TCGACCTCGA1980 - CTGGGAGTAC CCGAACGCCT GCGGCCTCAG CTGCGACAGC TCCGGTCCGG CCGCGCTGAA2040 - GAACATGGTC CAGGCGATGC GCGCCCAGTT CGGCACCGAC CTGGTCACCG CCGCCATCAC2100 - CGCCGACGCC AGCTCCGGCG GCAAGCTCGA CGCCGCCGAC TACGCGGGCG CCGCCCAGTA2160 - CTTCGACTGG TACAACGTGA TGACGTACGA CTTCTTCGGC GCCTGGGACA AGACCGGCCC2220 - GACCGCGCCC CACTCGGCCC TGAACTCCTA CAGCGGCATC CCCAAGGCCG ACTTCCACTC2280 - GGCCGCCGCC ATCGCCAAGC TCAAGGCGAA GGGCGTCCCG GCGAGCAAGC TCCTGCTCGG2340 - CATCGGCTTC TACGGCCGCG GCTGGACCGG CGTCACCCAG GACGCCCCGG GCGGCACCGC2400 - CACCGGCCCG GCCACCGGCA CCTACGAGGC GGGCATCGAG GACTACAAGG TCCTCAAGAA2460 - CACCTGCCCC GCCACCGGCA CCGTCGGCGG CACCGCGTAC GCCAAGTGCG GCAGCAACTG2520 - GTGGAGCTAC GACACCCCGG CCACCATCAA GACCAAGATG ACCTGGGCCA AGGACCAGGG2580 - CCTCGGCGGC GCCTTCTTCT GGGAGTTCAG CGGTGACACC GCGGGCGGCG AACTGGTCTC2640 - CGCGATGGAC TCCGGCCTCC GCTAGCCCCG GACCGGCACC CCGCCCGAAC CACTAGCACG2700 - ACCTCCCCCG GA2712

Fragments of the above chitinolytic enzymes are encompassed by the present invention.

Suitable fragments can be produced by several means. In the first, subclones of the gene encoding the chitinolytic enzymes of the present invention are produced by conventional molecular genetic manipulation by subcloning gene fragments. The subclones then are expressed in vitro or in vivo in bacterial cells to yield a smaller protein or peptide that can be tested for chitinolytic activity according to the procedure described below.

As an alternative, fragments of a chitinolytic enzyme can be produced by digestion of a full-length chitinolytic enzyme with proteolytic enzymes like chymotrypsin or Staphylococcus proteinase A, or trypsin. Different proteolytic enzymes are likely to cleave chitinolytic enzymes at different sites based on the amino acid sequence of the chitinolytic enzyme. Some of the fragments that result from proteolysis may be active chitinolytic enzymes.

In another approach, based on knowledge of the primary structure of the protein, fragments of a chitinolytic enzyme encoding gene may be synthesized by using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector for increased expression of a truncated peptide or protein.

Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences for a chitinolytic enzyme being produced. Alternatively, subjecting a full length chitinolytic enzyme to high temperatures and pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography, SDS-PAGE).

Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure and hydropathic nature of an enzyme. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

Suitable DNA molecules are those that hybridize to a DNA molecule comprising a nucleotide

sequence of SEQ. ID. Nos. 2 or 4 under stringent conditions. An example of suitable stringency conditions is when hybridization is carried out at 65.degree. C. for 20 hours in a medium containing 1M NaCl, 50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.1% sodium dodecyl sulfate, 0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 50 .mu.m g/ml E. coli DNA.

A chitinolytic enzyme of the present invention is preferably produced in purified form (preferably at least about 80%, more preferably 90%, pure) by conventional techniques. Typically, a chitinolytic enzyme of the present invention is secreted into the growth medium of recombinant host cells. Alternatively, a chitinolytic enzyme of the present invention is produced but not secreted into growth medium. In such cases, to isolate a chitinolytic enzyme, the host cell (e.g., E. coli) carrying a recombinant plasmid is propagated, lysed by sonication, heat, differential pressure, or chemical treatment, and the homogenate is centrifuged to remove bacterial debris. The supernatant is then subjected to sequential ammonium sulfate precipitation. The fraction containing a chitinolytic enzyme of the present invention is subjected to gel filtration in an appropriately sized dextran or polyacrylamide column to separate the proteins. If necessary, the protein fraction may be further purified by HPLC.

The DNA molecule encoding a chitinolytic enzyme can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Pat. No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccina virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK.+-. or KS.+-. (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif., which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F. W. Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems

infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promoters differ from those of procaryotic promoters. Furthermore, eucaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promoters are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

Promotors vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when cloning in E. coli, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, lac promotor, trp promotor, recA promotor, ribosomal RNA promotor, the P.sub.R and P.sub.L promoters of coliphage lambda and others, including but not limited, to lacUV5, ompF, bla, lpp, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid trp-lacUV5 (tac) promotor or other E. coli promoters produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promotor unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the lac operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as trp, pro, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promotor, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in E. coli requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the cro

gene or the N gene of coliphage lambda, or from the E. coli tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once an isolated DNA molecule encoding a chitinolytic enzyme has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like.

The present invention further relates to a method of effecting insect and/or fungus control for plants. This method involves applying a chitinolytic enzyme to all or part of a plant or a plant seed under conditions effective to control insects and/or fungi. Alternatively, the chitinolytic enzyme can be applied to plants such that seeds recovered from such plants themselves are able to effect insect and/or fungus control.

As an alternative to applying a chitinolytic enzyme to plants or plant seeds in order to control insects and/or fungi on the plants or plants grown from the seeds, transgenic plants or plant seeds can be utilized. When utilizing transgenic plants, this involves providing a transgenic plant transformed with a DNA molecule encoding a chitinolytic enzyme and growing the plant under conditions effective to permit that DNA molecule to control insects and/or fungi. Alternatively, a transgenic plant seed transformed with a DNA molecule encoding a chitinolytic enzyme can be provided and planted in soil. A plant is then propagated from the planted seed under conditions effective to control insects and/or fungi.

The embodiment of the present invention where the chitinolytic enzyme is applied to the plant or plant seed can be carried out in a number of ways, including: 1) application of an isolated chitinolytic enzyme and 2) application of bacteria which do not cause disease and are transformed with genes encoding a chitinolytic enzyme.

In one embodiment of the present invention, a chitinolytic enzyme of the present invention can be isolated (i.e. separated from the bacteria which naturally produce it) as described in the Examples infra. Preferably, however, an isolated chitinolytic enzyme of the present invention is produced recombinantly and purified (i.e. made substantially free of contaminants) as described supra.

In another embodiment of the present invention, a chitinolytic enzyme of the present invention can be applied to plants or plant seeds by applying bacteria containing genes encoding a chitinolytic enzyme. Such bacteria must be capable of secreting or exporting the enzyme so that the enzyme can effect fungus and/or insect control. In these embodiments, the chitinolytic enzyme is produced by the bacteria in planta or on seeds or just prior to introduction of the bacteria to the plants or plant seeds.

In the bacterial application mode of the present invention, the bacteria do not cause the disease and have been transformed (e.g., recombinantly) with a gene encoding a chitinolytic enzyme. For example, E. coli can be transformed with a gene encoding a chitinolytic enzyme and then applied to plants. Bacterial species other than E. coli can also be used in this embodiment of the present invention.

The method of the present invention can be utilized to treat a wide variety of plants or their seeds to control fungi and/or insects. Suitable plants include dicots and monocots. Monocots treatable in accordance with the present invention include Gramineae (e.g., grass, corn, grains, bamboo, sugar

cane), Liliaceae (e.g., onion, garlic, asparagus, tulips, hyacinths, day lily, and aloes), Iridaceae (e.g., iris, gladioli, freesia, crocus, and watsonia), and Orchidacea (e.g., orchid). Examples of dicots which can be treated pursuant to the present invention include Salicaceae (e.g., willow, and poplar), Ranunculaceae (e.g., Delphinium, Paeonia, Ranunculus, Anemone, Clematis, columbine, and marsh marigold), Magnoliaceae (e.g., tulip tree and Magnolia), Cruciferae (e.g., mustards, cabbage, cauliflower, broccoli, brussel sprouts, kale, kohlrabi, turnip, and radish), Rosaceae (e.g., strawberry, blackberry, peach, apple, pear, quince, cherry, almond, plum, apricot, and rose), Leguminosae (e.g., pea, bean, peanut, alfalfa, clover, vetch, redbud, broom, wisteria, lupine, black locust, and acacia), Malvaceae (e.g., cotton, okra, and mallow), Umbelliferae (e.g., carrot, parsley, parsnips, and hemlock), Labiatae (e.g., mint, peppermints, spearmint, thyme, sage, and lavender), Solanaceae (e.g., potato, tomato, pepper, eggplant, and Petunia), Cucurbitaceae (e.g., melon, squash, pumpkin, and cucumber), Compositae (e.g., sunflower, endive, artichoke, lettuce, safflower, aster, marigold, dandelions, sage brush, Dalia, Chrysanthemum, and Zinna), and Rubiaceae (e.g., coffee).

The present invention is effective against a wide variety of insect pests including the orders of Lepidoptera, Coleoptera, Diptera, Homoptera, Hemiptera, Thysanoptera, and Orthoptera. Examples of Lepidoptera include butterflies and moths. Coleoptera include beetles. Examples of Diptera are flies. Examples of Homoptera are aphids, whiteflies, scales, psyllids, leafhoppers, plant hoppers, cicadas, and treehoppers. The Hemiptera which are treatable in accordance with the present invention include true bugs. Thysanoptera which can be treated in accordance with the present invention include thrips. Examples of Orthoptera which can be treated in accordance with the present invention are grasshoppers, crickets, and katydids. Collectively, these orders of insect pests represent the most economically important group of pests for vegetable production worldwide.

The chitin-containing fungi inhibited by the purified chitinases of the present invention include, for example, species from the genera including Fusarium, Gliocadium, Rhizoctonia, Trichoderma, Uncinula, Ustilago, Erysiphe, Botrytis, Saccharomyces, Sclerotium, and Alternaria.

The method of the present invention involving application of a chitinolytic enzyme can be carried out through a variety of procedures when all or part of the plant is treated, including leaves, stems, roots, etc. This may (but need not) involve infiltration of the chitinolytic enzyme into the plant. Suitable application methods include topical application (e.g., high or low pressure spraying), injection, and leaf abrasion proximate to when enzyme application takes place. When treating plant seeds, in accordance with the application embodiment of the present invention, a chitinolytic enzyme can be applied by low or high pressure spraying, coating, immersion, or injection. Other suitable application procedures can be envisioned by those skilled in the art provided they are able to effect contact of the chitinolytic enzyme with the plant or plant seed. Once treated with a chitinolytic enzyme of the present invention, the seeds can be planted in natural or artificial soil and cultivated using conventional procedures to produce plants. After plants have been propagated from seeds treated in accordance with the present invention, the plants may be treated with one or more applications of a chitinolytic enzyme to control insects and/or fungi on the plants.

The chitobiosidase or endochitinase can be applied to plants or plant seeds in accordance with the present invention individually, in combination with one another, or in a mixture with other materials. Alternatively, a chitinolytic enzyme can be applied separately to plants with other materials being applied at different times.

A composition suitable for treating plants or plant seeds in accordance with the application embodiment of the present invention contains a chitinolytic enzyme in a carrier. Suitable carriers include water, aqueous solutions, slurries, or dry powders. In this embodiment, the composition

contains greater than 500 nM chitinolytic enzyme.

Although not required, this composition may contain additional additives including fertilizer, insecticide, fungicide, nematacide, and mixtures thereof. Suitable fertilizers include (NH.sub.4).sub.2 NO.sub.3. An example of a suitable insecticide is Malathion. Useful fungicides include Captan.

Other suitable additives include buffering agents, wetting agents, coating agents, and abrading agents. These materials can be used to facilitate the process of the present invention. In addition, a chitinolytic enzyme can be applied to plant seeds with other conventional seed formulation and treatment materials, including clays and polysaccharides.

In the alternative embodiment of the present invention involving the use of transgenic plants and transgenic seeds, a chitinolytic enzyme need not be applied topically to the plants or seeds. Instead, transgenic plants transformed with a DNA molecule encoding a chitinolytic enzyme are produced according to procedures well known in the art.

In producing transgenic plants, the DNA construct in a vector described above can be microinjected directly into plant cells by use of micropipettes to transfer mechanically the recombinant DNA.. Crossway, Mol. Gen. Genetics, 202:179-85 (1985), which is hereby incorporated by reference. The genetic material may also be transferred into the plant cell using polyethylene glycol. Krens, et al., Nature, 296:72-74 (1982), which is hereby incorporated by reference.

Another approach to transforming plant cells with the DNA construct is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Pat. Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, a vector containing the DNA construct can be introduced into the cell by coating the particles with the vector containing that heterologous DNA construct. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the vector and heterologous DNA construct) can also be propelled into plant cells.

Yet another method of introduction is fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies. Fraley, et al., Proc. Natl. Acad. Sci. USA, 79:1859-63 (1982), which is hereby incorporated by reference.

The DNA molecule may also be introduced into the plant cells by electroporation. Fromm et al., Proc. Natl. Acad. Sci. USA, 82:5824 (1985), which is hereby incorporated by reference. In this technique, plant protoplasts are electroporated in the presence of plasmids containing the expression cassette. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and regenerate.

Another method of introducing the DNA molecule into plant cells is to infect a plant cell with Agrobacterium tumefaciens or A. rhizogenes previously transformed with the gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants. Generally, this procedure involves inoculating the plant tissue with a

suspension of bacteria and incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28.degree. C.

Agrobacterium is a representative genus of the gram-negative family Rhizobiaceae. Its species are responsible for crown gall (A. tumefaciens) and hairy root disease (A. rhizogenes). The plant cells in crown gall tumors and hairy roots are induced to produce amino acid derivatives known as opines, which are catabolized only by the bacteria. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In addition, assaying for the presence of opines can be used to identify transformed tissue.

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of A. tumefaciens or the Ri plasmid of A. rhizogenes. The Ti or Ri plasmid is transmitted to plant cells on infection by Agrobacterium and is stably integrated into the plant genome. J. Schell, Science, 237:1176-83 (1987), which is hereby incorporated by reference.

After transformation, the transformed plant cells must be regenerated.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I. R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. III (1986), which are hereby incorporated by reference.

It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugarcane, sugar beets, cotton, fruit trees, and legumes.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts or a petri plate containing explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

After the expression cassette is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Once transgenic plants of this type are produced, the plants themselves can be cultivated in accordance with conventional procedure so that the DNA construct is present in the resulting plants. Alternatively, transgenic seeds are recovered from the transgenic plants. These seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic plants.

When transgenic plants and plant seeds are used in accordance with the present invention, they additionally can be treated with the same materials as are used to treat the plants and seeds to which a chitinolytic enzyme is applied. These other materials, including a chitinolytic enzyme, can be applied to the transgenic plants and plant seeds by the above-noted procedures, including high or low pressure spraying, injection, coating, and immersion. Similarly, after plants have been propagated from the transgenic plant seeds, the plants may be treated with one or more applications of a

chitinolytic enzyme to control fungi and/or insects. Such plants may also be treated with conventional plant treatment agents (e.g., insecticides, fertilizers, etc.). The transgenic plants of the present invention are useful in producing seeds or propagules (e.g., cuttings) from which fungus and/or insect resistant plants grow.

EXAMPLES

Example 1

Bacteria

Bacterial strains were grown on slants of trypticase soy agar (BBL, Cockeysville, Md.) for maintenance of cultures and production of inoculum. Growth from agar slants was transferred to a liquid medium consisting of 50 mM magnesium sulfate, 0.1% glucose, 0.1% calcium chloride, 0.05% manganese sulfate, 0.025% ferrous sulfate, 0.00125% zinc sulfate, and 0.5% crab shell chitin. The cultures were grown in flasks with constant shaking at 30 C for 4-5 days, or in an 18 liter fermenter (Microfilm Model MF 214, New Brunswick Scientific Co., New Brunswick N.J.) at 30-30 C, air flow of 200-400 cc min.sup.-1, and impeller speed of 150 rpm for 5 days.

Example 2

Survey of Chitinolytic Enzymes with Activity in an Alkaline Environment

Over 100 strains of microorganisms from alkaline soil were screened for their ability to clear chitin-containing agar plates buffered at pH 9. From this survey, a strain of Streptomyces was isolated that secreted chitinolytic enzymes which were active at pH 9, when the strain was grown on chitin-containing agar plates (pH 9) or liquid culture medium (pH 9) containing chitin.

Example 3

Characterization of Active Strain

The active Streptomyces species was evaluated by the standard taxonomic criteria of the International Streptomyces Project (Shirling, et al., "Methods for Characterization of Streptomyces Species," Int. J. Syst. Bacteriol., 16:313-40 (1966), which is hereby incorporated by reference), including (a) growth on yeast extract-malt extract agar, inorganic salts-starch agar, glycerol-asparagine agar and Czapek's agar, (b) production of melanin pigments, and (c) utilization of 11 different carbohydrates as the sole carbon source. The strain was also grown for 4 days at 28 C on trypticase soy broth agar plates, the biomass scraped from the plates. Saponification of the cells and methylation of the fatty acids were done according to the method of Korn-Wendisch et al. (Korn-Wendisch, et al., "Transfer of Faenia rectivirgula Kurup and Agre 1983 to the Genus Saccharopolyspora Lacey and Goodfellow 1975, Elevation of Saccharopolyspora hirsuta subsp. taberi Labeda 1987 to Species Level, and Emended Description of the Genus Saccharopolyspora," Int. J. Syst. Bacteriol., 39:430-41 (1989), which is hereby incorporated by reference), and the profile of fatty acid methyl esters present in the extracts was determined by gas chromatography using an HP 5890A gas chromatograph fitted with 25 m.times.0.2 mm phenyl methyl silicone fused silica capillary column (Hewlett-Packard, Inc., Palo Alto, Calif.) and Microbial Identification System software (Microbial ID, Inc., Newark, Del.), using the standard calibration mixture provided by the manufacturer. The fatty acid methyl ester profile observed for this strain was compared with those in the ACTINO library provided with the Microbial Identification System, as well as with in-house STMYB9 Streptomyces profile library.

The active strain was identified as a member of Streptomyces albidoflavus, based of morphological and physiological characteristics, and fatty acid profile (Table 1).

TABLE 1

Comparison of fatty acid profiles between Streptomyces strain NRRL B-16746 and that of the library entry for Streptomyces albidoflavus.

Fatty Acid	Library Entry S. albidoflavus	NRRL B-16746 Streptomyces sp.
14:0 iso	5.92-25.81 6.47	
15:0 iso	2.50-9.50	7.38
15:0 anteiso	6.74-29.26	26.35
15:1 B	0.00-5.55	2.55
15:0	2.55-10.80	3.84
16:1 iso H	3.30-18.70	4.99
16:1 iso	14.84-33.30	18.26
16:1 cis 9	1.40-8.06	4.83
15:0 anteiso 2-hydroxyl	1.35-8.68	3.12
16:0	1.41-6.39	5.17
16:0 9? methyl	0.00-3.59	2.32
17:1 anteisi C	1.01-4.30	3.76
17:0 iso	0.00-2.04	1.81
17:0 anteiso	1.11-7.08	7.50
17:1 cis 9	0.00-2.99	1.64
16:0 iso 2-hydroxyl	0.00-6.85	0.00

*There was a 0.319 probability of matching S. albidoflavus using the ACTINO library supplied by MIDI, and a 0.764 probability of match using the inhouse STMYB9 (Streptomyces Bergey's Manual, 9th Edition) library.

The strain was characterized as having olive-buff, smooth surfaced spores borne on flexuous sporophores and moderate yellowish-brown vegetative mycelium, was non-chromogenic, and utilized glucose, D-xylose, L-arabinose, D-fructose, D-galactose, raffinose, D-mannitol, inositol, salicin, and sucrose, but did not utilize L-rhamnose as sole carbon source. The strain was accessioned into the ARS Culture Collection (Peoria, Ill.) as NRRL B-16746.

The ability of streptomycetes to degrade chitin is well documented (Clarke, P. H., "The Occurrence of Chitinase in Some Bacteria," J. Gen. Microbiol., 14:188-96 (1956), which is hereby incorporated by reference) and is characteristic of about 25% of the members of this genus (Williams, et al., "Numerical Classification of Streptomyces and Related Genera," J. Gen. Microbiol., 129:1743-1813 (1983), which is hereby incorporated by reference). Streptomycetes have been shown to play a major role in degradation of chitin from fungal mycelium in acidic soil and litter (Williams, et al., "The Role of Streptomycetes in Decomposition of Chitin in Acidic Soils," J. Gen. Microbiol., 127:55-63 (1981), which is hereby incorporated by reference).

Example 4

In Vitro Production of Alkaline Chitinolytic Enzymes

In general, secretion of fungal and bacterial chitinolytic enzymes is regulated by availability of carbon (e.g., chitodextrins); the presence of glucose or N-acetylglucosamine represses secretion of chitinolytic enzymes, while chitin induces the secretion of these enzymes (Harman, et al., "Chitinolytic Enzymes of Trichoderma harzianum: Purification of Chitobiase and Endochitinase," Phytopathology, 83:313-18 (1993), Monreal, et al., "The Chitinase of Serratia Marcescens," Can. J. Microbiol., 15:689-96 (1969), St. Leger, et al., "Cuticle-Degrading Enzymes of Entomopathogenic Fungi: Regulation of Production of Chitinolytic Enzymes," J. Gen. Microbiol., 132:1509-17 (1986), Ulhoa, et al., "Regulation of Chitinase Synthesis in Trichoderma harzianum," J. Gen. Microbiol., 137:2163-69 (1991), which are hereby incorporated by reference). In support of these previous reports, the presence of chitin in the liquid medium resulted in a minimum of a 5-fold increase of all three types of enzyme activity secreted by S. albidoflavus (Table 2).

TABLE 2

The influence of chitin amendment on the level of chitinolytic activity secreted by Streptomyces albidoflavus NRRL B-16746.

	Glucosaminidase		Chitobiosidase		Endochitinase	
	% nkatals		% nkatals		Units/ml	
Broth*	pH 5	pH 9	pH 5	pH 9	pH 5	pH 9
+ chitin	87 .+-. 20	12 .+-. 9	51 .+-. 11	24 .+-. 18	79	43 .+-. 2
- chitin	16 .+-. 5	2 .+-. 1.6	7 .+-. 1	1 .+-. 0.5	0	0

*+ chitin indicates 0.5% chitin + 0.1% glucose in the standard growth medium; - chitin indicates 0.3% glucose and no chitin. Data are the average .+-. SE from three replicates.

Based on these findings, chitin was added to the liquid medium to enhance the production of chitinolytic enzymes by S. albidoflavus NRRL B-16746. Addition of glucose also influenced the level of chitinolytic enzyme activity (FIG. 1); however, this was not always in the suppressive manner reported previously. After 6 days of culture, low levels of glucose (i.e., 0.01%) elevated the level of chitinolytic activity by 10-20%, while high levels of glucose (i.e., 0.5%) suppressed chitinolytic activity by 50-75%.

In conjunction with this latter study, the time course of production of chitinolytic enzymes by S. albidoflavus NRRL B-16746 was examined. Polyacrylamide gel electrophoresis ("PAGE") followed by Coomassie staining indicated that a limited number of proteins occurred in the broth during 8 days of culture (FIG. 2A). A comparison of this gel with PAGE followed by agar-overlay containing a chitinolytic enzyme-specific, fluorogenic substrate indicated that 50-75% of the protein in the broth had chitinolytic activity (FIG. 2B). Furthermore, comparison of the two gels (FIGS. 2A and B) indicated that the enzymes responsible for chitinolytic activity were differentially induced. The proteins with chitobiosidase and glucosaminidase activity were detectable at 96 hrs, while a single endochitinase was detectable after 72 hrs of culture, suggesting that the endochitinase was regulated independently from the other chitinolytic enzymes. Moreover, two additional endochitinases were

detectable at 96 hrs, suggesting that these endochitinases may be regulated independently from each other. The more prominent of the two later endochitinases was larger and/or more basic than the first endochitinase, suggesting that these two proteins were encoded by different genes. The less prominent of the two later endochitinases was smaller and/or more acidic than the other two proteins. This third protein may have been the result of a third gene, or post-translational modification (e.g., proteolytic digestion) of the previous protein(s). In support of the latter possibility, proteolytic activity was detectable in the culture filtrate, and this enzyme activity was inhibited 45-70% by serine proteinase inhibitors.

Example 5

In vitro Analyses of Enzymatic Activity

Endochitinase activity was measured at pH 3-12, or pH 5 and pH 9. A 500 .mu.l aliquot of sample was mixed with 500 .mu.l 50 mM Tris pH 9, 0.1 M acetate buffer pH 5, or 0.1 M citrate-phosphate buffer pH 3-12 (for pH optimum study) containing 4% colloidal chitin. The mixture was shaken at 30 C for 24 hr. 5 ml of dH.sub.2 O was then added to each tube, vortexed, and the optical density was measured at 510 nm. Percent reduction of turbidity was calculated for each tube. For calculation of specific activity, one unit was defined as the amount of enzyme required to obtain 1% reduction of turbidity under the above conditions.

Chitobiosidase activity was measured at pH 3-12, or pH 5 and pH 9. A 30 .mu.l aliquot of sample was transferred to a microtiter plate, and 50 .mu.l of 50 mM Tris pH 9, 0.1 M acetate buffer pH 5, or 0.1 M citrate-phosphate buffer pH 3-12 (for pH optimum study) containing 0.03% p-nitrophenyl-D-N,N'-diacetylchitobiose was added to each well. The plate was incubated at 50 C for 15 min, then 50 .mu.l of 0.4 M sodium carbonate was added to each well, and the optical density was measured at 410 nm. One nanokatal (nkatal) corresponds to the release of 1 nmol nitrophenyl.times.s.sup.-1 under the above conditions. Data are reported as % nkatals (i.e., nkatals/100 ml).

Glucosaminidase activity was measured following the same procedure as that for chitobiosidase except the substrate was p-nitrophenyl N-acetyl-.beta.-D-glucosaminide.

Prior to protein analyses, each fraction was dialyzed against dH.sub.2 O for 30 hr, to remove salts. Total protein was determined for each fraction using Coomassie stain reagent (Pierce Chemical Co., Rockford, Ill.). Chitinase from Serratia marcescens (Sigma Chemical Co., St. Louis, Mo.) was used as a standard.

Example 6

pH Range of Activity of Alkaline Chitinolytic Enzymes

Following 4-5 days of culture of S. albidoflavus in liquid medium containing chitin, the broth was filter-sterilized, dialyzed (MWCO12,000-14,000), and concentrated. This solution was used to determine the pH optimum for glucosaminidase, chitobiosidase, and endochitinase activity (FIG. 3). Although these enzymes had optimal activity at pH 4-6, there was significant endochitinase and chitobiosidase activity at pH 8-9. When comparing enzyme activity at pH 9 with activity at pH 5 from six different preparations of bacterial broth supernatant, only 6.8.+-.1.6% of glucosaminidase activity was detectable at pH 9 compared to pH 5, while 54.5.+-.9.7% of the chitobiosidase activity was detectable and 74.4.+-.8.1% of the endochitinase activity was detectable. The chitinolytic enzymes from other species of Streptomyces retain activity in alkaline conditions; however, the level of

activity at pH 9 generally is 10-40% of the activity at pH 5 (Hara et al., "Purification and Characterization of Chitinase Produced by Streptomyces erythraeus," J. Biochem., 105:484-89 (1989), Neugebauer, et al., "Chitinolytic Properties of Streptomyces lividans," Arch. Microbiol, 156:192-97 (1991), Tsujibo, et al., "Purification and Properties of Two Types of Chitinases Produced by an Alkalophilic Actinomycete," Biosci. Biotech. Biochem., 56:1304-05 (1992), Ueno, et al., "Purification and Some Properties of Extracellular Chitinases from Streptomyces sp. S-84," J. Gen. Appl. Microbiol., 36:377-92 (1990), Yabuki, et al., "Purification and Characterization of Chitinase and Chitobiase Produced by Aeromonas hydrophila subsp. anaerogenes A52," J. Gen. Anpl. Microbiol., 32:25-38 (1986), which are hereby incorporated by reference). An exception is a thermophilic bacteria, Streptomyces thermoviolaceus, that secretes an endochitinase that has optimal activity at pH 8-10. However, this enzyme activity requires an environmental temperature of 70-80 C (Tsujibo, et al., "Purification and Properties of a Thermostable Chitinase from Streptomyces thermoviolaceus OPC-520," Appl. Environ. Microbiol., 59:620-22 (1993), which is hereby incorporated by reference). Since chitinolytic enzymes that functioned in an alkaline environment were of interest, the chitobiosidases and endochitinases were characterized.

Example 7

Time Course of Enzyme Secretion

Liquid medium was prepared with either 0.3% glucose or 0.5% chitin+0%, 0.1%, 0.25%, or 0.5% glucose. A 10 ml aliquot was removed from the bacterial shaker flask every day for 8 days. On each day of collection, each sample was centrifuged and filtered, then each sample was adjusted to contain 0.02% sodium azide (anti-microbial agent). After all samples were collected, they were analyzed for enzyme activity. To prepare the samples for electrophoresis, they were dialyzed against dH.sub.2 O to remove all salt, and concentrated in a speed-vac (Savant).

Example 8

Purification of Chitinolytic Enzymes

The strain of Streptomyces albidoflavus used for production of chitinolytic enzymes is accessioned in the ARS Culture Collection (Peoria, Ill.) as NRRL B-16746 (Broadway, et al., "Partial Characterization of Chitinolytic Enzymes from Streptomyces albidoflavus," Lett. App. Microbiol., 20:271-76 (1995), which is hereby incorporated by reference). Chitinolytic enzymes were produced by Streptomyces albidoflavus when grown in liquid medium containing 0.012% magnesium sulfate, 0.1% glucose, 0.1% calcium chloride, 0.05% manganese sulfate, 0.025% ferrous sulfate, 0.00125% zinc sulfate, and 0.5% crab shell chitin in 50 mM Tris, pH 9.0. Cultures were grown in flasks with constant shaking (250 rpm) at 30 C for 4-5 days. The biomass was removed from the broth by centrifugation at 6000.times.g for 30 min, 4 C. The supernatant was filtered through mira cloth, then adjusted to 95% saturation with ammonium sulfate to isolate total protein. Ammonium sulfate precipitation enhanced the relative level of activity of the chitobiosidases and endochitinases, and reduced the level of glucosaminidase activity that was detectable at pH 9 (Table 3).

TABLE 3

	Glucosaminidase		Chitobiosidase		Endochitinase	
	% nkatals	% nkatals	Units/ml			
Broth*	pH 5	pH 9	pH 5	pH 9	pH 5	pH 9

Broth	131	10	81	60	29	0
AmmSO.sub.4 precipitate	63	5	81	75	41	23
AmmSO.sub.4 supernatant	1	1	14	2	18	0

The ammonium sulfate ("AmmSO.sub.4 ") precipitate was incubated at 4 C, overnight, then centrifuged at 6000.times.g for 30 min. at 4 C. The pellet was resuspended in dH.sub.2 O and dialyzed against ice-cold dH.sub.2 O (130.times.vol) to remove salt. The dialysate was centrifuged at 6000.times.g for 10 min, 4 C to remove insoluble particles, and the supernatant was lyophilized. This powder was identified as semipurified chitinolytic enzyme mixture.

A 50 mg sample of the lyophilized protein was resuspended in 100 mM Tris pH 8.5, then applied to a low pressure anion exchange column (16 cm.times.2.5 cm DEAE, Pharmacia) that was equilibrated with Tris buffer, pH 8.5. Fractions of 8 ml were collected (FIG. 4). Non-adsorbed material (peak I, which included endochitinases) was washed from the column with the Tris buffer until the optical density of the eluent approached zero. The chitobiosidases were then eluted with peak II with Tris buffer containing 0.2 M NaCl. The column was then cleaned with Tris buffer containing 1.0 M NaCl. The endochitinase (peak 1 from the DEAE column) was further purified by perfusion chromatography (BioCAD Sprint, Perceptive) on an HQ/M anion exchange column (4.6 mm.times.100 mm), equilibrated with 20 mM Tris/bis-Tris propane, pH 9.0. The sample was dialyzed against ice-cold dH.sub.2 O, then applied to the column; fractions of 2 ml were collected. Non-adsorbed material was washed from the column with buffer, then endochitinases were eluted with a gradient of sodium chloride (0 to 100 mM). The column was cleaned with buffer containing 1.0 M NaCl. Anion exchange chromatography purified the chitobiosidase 12.6.times., and the endochitinase 13.5.times. (Table 4).

TABLE 4

Purification of chitinolytic enzymes in broth from Streptomyces albidoflavus NRRL B-16746. All enzyme assays were performed at pH 9.

Purification Step	Total Activity (units)	Specific Activity (units/mg)	Protein (%)	Yield	Purification Factor
Culture filtrate					
chitobiosidase	140	0.5	100		1.0
endochitinase	18,600	67	100		1.0
Ammonium sulfate ppt					
chitobiosidase	137	1.2	98	2.4	
endochitinase	7735	68	42	1.0	
DEAE Column					
chitobiosidase	107	6.3	76	12.6	
endochitinase	9045	905	49	13.5	

An alternative to the DEAE anion exchange chromatography for separating the endochitinases from the chitobiosidases in the ammonium sulfate precipitate is by the direct use of perfusion

chromatography (BioCAD Sprint, PerSeptive Biosystems, Cambridge, Mass.) on an HG/M strong anion exchange column (4.6 mm.times.100 mm), equilibrated with 20 mM Tris/bis-Tris propane, pH 9.0. A 5 mg sample of chitinolytic enzyme mixture was applied to the column, and the endochitinases were separated from the chitobiosidases, as shown by the elution profile of the column (FIG. 9). The endochitinases were eluted as 2 protein peaks: peak I was eluted with the Tris buffer and peak II was eluted with 80 mM NaCl in Tris buffer. The chitobiosidases were eluted with 300 mM NaCl in Tris buffer. The column was cleaned with buffer containing 2 M NaCl. Samples were collected in 2 ml fractions; fractions were pooled to combine protein(s) from a single peak. Each peak of protein was dialyzed against ice-cold dH.sub.2 O, lyophilized, then analyzed for total protein and enzyme activity. The proteins were then applied to a non-denaturing polyacrylamide PhastGel (procedure described below) to confirm the presence of endochitinase(s) and/or chitobiosidase(s) (FIG. 10A). Based on these in vitro analyses, peaks I and II contained endochitinase activity, peaks IV and V had chitobiosidase activity, while peak III had endochitinase and chitobiosidase activity. Peak III was not used for bioassays against insects, because it contained both types of enzyme activity]. Peak VI had no chitinolytic activity (FIG. 10B).

Example 9

Polyacrylamide Gel Electrophoretic Analyses of Chitinolytic Enzymes

The PhastSystem electrophoresis unit (Pharmacia, Uppsala, Sweden) was used to characterize the proteins with chitinolytic activity. The number of proteins with chitinolytic activity was determined on a non-denaturing, discontinuous polyacrylamide gel (7.5% stacking gel, 20% separating gel, separation length 32 mm), using non-denaturing buffer strips containing 0.88 M L-alanine, 0.25 M Tris, pH 8.8. A 3 .mu.l aliquot of the sample was mixed with 1 .mu.l 4.times.sample buffer (0.25 M Tris, pH 8.8, 0.008% bromophenol blue (w/v)), then transferred to a sample applicator for electrophoresis.

The molecular weights were determined on 0.45 mm discontinuous, polyacrylamide PhastGel consisting of a 20% polyacrylamide separating gel and a 7.5% polyacrylamide stacking gel with a separating length of 32 mm. The buffer strips contained 0.2 M Tricine, 0.2 M Tris, 0.55% SDS, pH 8.1. A 3 .mu.l aliquot of the sample was mixed with 1 .mu.l 4.times. sample buffer (40 mM Tris, 4 mM EDTA, pH 8.0, 10% SDS, 20% .beta.-mercaptoethanol, 0.04% bromophenol blue (w/v)), then placed in boiling water for 30 sec, centrifuged for 15 sec, and transferred to a sample applicator for electrophoresis. The molecular weight markers (BioRad) included soybean trypsin inhibitor (21.5 kD), carbonic anhydrase (31 kD), ovalbumin (45 kD), bovine serum albumin (66.2 kD), and phosphorylase B (97.4 kD).

Isoelectric points were determined on a 0.35 mm 5% polyacrylamide PhastGel containing ampholytes for pI 4 to 6.5 with a total separation length of 37 mm. A 4 .mu.l aliquot of each sample was analyzed. A mixture of isoelectric focusing markers (Sigma) included methyl red marker dye (pI 3.8), amyloglucosidase (pI 3.6), soybean trypsin inhibitor (pI 4.6), bovine beta-lactoglobulin A (pI 5.1), carbonic anhydrase II (pI 5.9), carbonic anhydrase I (pI 6.6), horse myoglobin (pI 6.8 and 7.2), L-lactic dehydrogenase (pI 8.3, 8.4, and 8.6), and bovine trypsinogen (pI 9.3).

All protein bands were visualized by staining the polyacrylamide gel with Coomassie (0.1% Coomassie R350 in 30% MeOH, 10% acetic acid). Proteins with chitobiosidase and endochitinase activity were detected on gels with an overlay containing 0.025% 4-methylumbelliferyl .beta.-D-N,N'-diacetylchitobioside in 0.05 M Tris pH 9, 1% low melting DNA-grade agarose. Glucosaminidase activity was detected on polyacrylamide gels with an overlay containing 0.025% 4-

methylumbelliferyl-N-acetyl-.beta.-D-glucosaminide. The agarose-based mixture was boiled for 5 min, then cooled to 35 C. Immediately following electrophoresis, the agarose mixture was poured over the gel, and the fluorescent bands were visualized with UV light.

Polyacrylamide gel electrophoresis ("PAGE") followed by Coomassie staining indicated that the broth from Streptomyces albidoflavus contained a limited number of proteins. PAGE followed by agar-overlay of an enzyme-specific, fluorogenic substrate (Harman, et al., "Chitinolytic Enzymes of Trichoderma harzianum: Purification of Chitobiase and Endochitinase," Phytopathology, 83:313-18 (1993), which is hereby incorporated by reference) indicated that 50-75% of the proteins in the broth had chitinolytic activity (Broadway, et al., "Partial Characterization of Chitinolytic Enzymes from Streptomyces albidoflavus," Lett. Appl. Microbiol., 20:271-76 (1995), which is hereby incorporated by reference).

Each protein peak from the DEAE column was dialyzed against dH.sub.2 O, then concentrated 10.times. in a speed-vac (Savant) prior to electrophoretic analyses. PAGE of the proteins in peak II indicated that the chitobiosidase activity originated from two proteins (FIG. 5) with isoelectric points 3.0 (FIG. 6), and molecular weights of 27 and 34 kD (FIG. 7). Perfusion chromatography of the endochitinases (peak I from DEAE) removed contaminating proteins. Endochitinase activity occurred in peaks III and IV (FIG. 8). PAGE of the proteins in these two peaks indicated that the endochitinase activity originated from 5 major proteins (FIG. 5) with isoelectric points of 5.1, 5.3, 5.75, 5.8-5.9, and 6.4 (FIG. 6), and a minor protein at pI 5.0. The molecular mass of the 5 major endochitinases were 59, 45, 38.5, 27, and 25.5 kD (FIG. 7). This study demonstrates that the chitinolytic enzymes from S. albidoflavus NRRL B-16746 have activity at the pH that occurs in the guts of herbivorous insects, and are small enough to allow molecular transformation of agricultural crops.

Example 10

Glycosylation

A glycan detection kit (Boehringer Mannheim, Indianapolis, Ind.) was used to determine whether the chitinolytic enzymes were glycosylated. In brief, the proteins were oxidized with periodate, then incubated with digoxigenin, which binds to oxidized sugar moieties. The mixture was electrophoresed on SDS-PAGE, transferred to nitrocellulose, then incubated with anti-digoxigenin antibody, which is conjugated to alkaline phosphatase. The antibody-glycoprotein complexes were visualized calorimetrically.

Digoxigenin glycan analysis (Boehringer Mannheim Biochemica) indicated that the chitinolytic enzymes were not glycosylated.

Example 11

Amino Acid Analysis

Amino acid analyses were performed at the Cornell Amino Acid Facility on the Waters Pico-Tag HPLC System (Cohen et al. "Analysis of Amino Acids Using Pre-column Derivatization with Phenylisothiocyanate," Amer. Lab, (1984), which is hereby incorporated by reference). Samples were concentrated, then the endochitinases were separated by native-PAGE, and the chitobiosidases were separated by SDS-PAGE. The proteins were electrophoretically transferred to PVDF transfer membrane (Immobilon-P, 0.45 .mu.m, Millipore) using the PhastSystem Transfer Unit and Tris/glycine transfer buffer (25 mM Tris, 192 mM glycine, pH 8.5, 20% MeOH). The Immobilon was

stained with Coomassie (0.1% Coomassie, 60% MeOH, 7.5% acetic acid), destained with 75% MeOH, 7.5% acetic acid, then exhaustively rinsed with double distilled H.sub.2 O to remove excess glycine originating from the transfer buffer. Each protein band was acid hydrolyzed (6N HCl, 115 min, 150, under partial vacuum and N.sub.2), the amino acids were extracted from the Immobilon, then analyzed for amino acid composition (Cohen et al. "Analysis of Amino Acids Using Pre-column Derivatization with Phenylisothiocyanate," Amer. Lab, (1984), which is hereby incorporated by reference).

Amino acid analysis was performed on the two chitobiosidase proteins and three of the endochitinase proteins (Table 5).

TABLE 5

Amino acid analysis of five chitinolytic enzymes from Streptomyces albidoflavis NRRL B-16746.

	Chitobiosidases		Endochitinases		
	A	B	C	D	E
	34 kD	27.5 kD pI	6.4 pI	5.75 pI	5.3
Amino Acid	% pmol				
ASX	13	12	9	10	9
GLX	9	9	5	5	5
HIS	1	4	2	2	2
ARG	3	4	3	3	4
LYS	3	2	7	7	5
SER	6	7	6	6	7
GLY	11	14	13	13	13
THR	8	7	9	9	10
TYR	4	5	5	5	4
CYS	0	1	1	0	1
MET	2	1	1	1	1
ALA	14	10	15	14	16
PRO	4	6	5	5	6
VAL	7	5	5	5	7
ILE	3	3	4	3	4
LEU	8	7	6	6	6
PHE	4	5	5	5	4

The chitinolytic enzymes had 40.6.+-.1.4% pmol of hydrophobic amino acids, indicating that these enzymes were not membrane proteins (membrane-bound proteins contain more than 30-40% hydrophobic amino acids), and they contained 7-12% pmol of basic amino acids, which is comparable to generalized proteins (Reeck, G. "Proteins" in CRC Handbook of Biochemistry and Moleuclar Biology, pp. 504-511, Edited by G. D. Fasman. Cleveland: CRC Press (1976), which is hereby incorporated by reference). However, the chitobiosidases had a high level of acidic amino acids (i.e., 21-22%) compared to the endochitinases (14-15% acidic amino acids), which accounts for the strongly acidic isoelectric point of the chitobiosidases.

Example 12

Insects

To determine the effect of chitinolytic enzymes on the growth and development of Lepidoptera, larval

Trichoplusia ni (cabbage looper) were reared on a high wheat germ-based meridic diet (Webb et al., "Laboratory Rearing of the Imported Cabbageworm," NY Food Life Sci. Bull., 122 (1988), which is hereby incorporated by reference) supplemented with chitinolytic enzymes. Each bioassay included 4 treatments (0, 0.25%, 0.5%, and 1% of chitinolytic enzyme mixture), 3 cups/treatment, 20 neonate larvae/cup, and each bioassay was replicated three times. All larvae were weighed when controls reached the ultimate instar and then monitored daily for developmental changes. The percent pupation and percent adult emergence was based on the total number of larvae weighed and total number of pupae recovered from each test diet, respectively.

Larvae of the Coleoptera Hypothenemus hampei (coffee berry borer) were maintained individually in wells of an ELISA plate, each well containing 0.2 ml of artificial diet (Villacorta et al., "Nova Dieta Meridica Para Criacao De Hypothenemus hampei (Ferrari) (Coleoptera: Scolytidae)," A. Soc. Entom. Brasil, 22:405-09 (1993), which is hereby incorporated by reference). Incubation conditions were maintained at 26 C, 60-70% relative humidity. Each bioassay included 4 treatments (0, 0.25%, 0.5%, and 1% chitinolytic enzyme mixture), 20 eggs/treatment, and each bioassay was replicated three times. The insects were monitored during 30 days for developmental changes (from egg to adult) and mortality.

The Homoptera Myzus persicae (green peach aphid) was reared on turnip plants prior to use in experiments. Each bioassay consisted of 6 treatments (0%, 0.06%, 0.125%, 0.25%, 0.5%, and 1% chitinolytic enzyme mixture prepared in 20% sucrose), replicated four times each. Immature wingless aphids of similar age, were each placed in a glass cylinder arena (22 mm inner diameter, 21 mm height) covered on one end with parafilm. The other end of the arena was sealed when placed upright on a supporting substrate. An aliquot of 20% sucrose solution was added to the top of the parafilm, and another piece of parafilm stretched over the solution to form a thin layer of solution. The parafilm provided a membrane through which aphids could feed. Aphids were held for 24 hrs. in the cylinders to become acclimated to the test arena. After 24 hours, the number of surviving aphids was recorded, the parafilm was replaced, and the sucrose solution was replaced with experimental chitinase solutions. Percent mortality was recorded at 24 and 48 hrs. after the chitinase solutions were added.

Adult whiteflies, Bemisia argentifolii, of similar age were obtained by placing poinsettia leaves infested with pupae from the colony into sleeve cages provisioned with a poinsettia plant. Adults were allowed to emerge from pupae for one day before the remaining pupae were removed to prevent further adult emergence. Adults were then left in the cage on the poinsettia plant for an additional two days before use. Bioassays were conducted within polycarbonate vials (45 mm diameter, 74 mm height) over which parafilm was thinly stretched. A 0.5 ml aliquot of 10% sucrose solution, with or without chitinolytic enzymes, was placed onto the parafilm and covered by another tightly stretched layer of parafilm as with aphid trials. Twenty adult whiteflies were released into each vial through a small hole in the side of the vial and periodically evaluated for mortality over 3 d. Bioassays included a control treatment (10% sucrose) and either semipurified chitinase at 0.06, 0.125, 0.25, 0.5, and 1.0%, or one of five chitinase fractions at 0.5%. Each treatment was replicated three to six times for each bioassay. Aphid and whitefly trials were conducted on a laboratory bench under conditions of 12L:12D and 22C.

Example 13

Biological Activity of Alkaline Chitinolytic enzymes Against Insects

In vitro analyses indicated that chitinolytic enzymes will digest the peritrophic membrane from larval Lepidoptera. In addition, there was no loss of chitinolytic activity when the contents within the

midgut lumen from larval Lepidoptera (Trichoplusia ni or Pieris rapae) was incubated for 60 min with a crude mixture of chitinolytic enzymes from Streptomyces albidoflavus, indicating that the bacterial chitinolytic enzymes were not susceptible to proteolytic digestion by the enzymes in the midgut lumen of larval T. ni and Pieris rapae. Also, chitinolytic enzymes did not lose activity when incorporated into an artificial diet and maintained in an environmental chamber for 5 days, or when ingested by larval T. ni, indicating that the enzymes could be incorporated into an artificial diet for biological testing, and that insects did not secrete an inhibitor of chitinolytic enzymes when there was chitinolytic activity in the gut. More importantly, a mixture of chitobiosidases and endochitinases (extracted from the bacterial culture filtrate by ammonium sulfate precipitate, then dialyzed, and lyophilized) significantly reduced the growth and/or survival of herbivorous insects (as shown below).

The lyophilized, chitinolytic enzyme mixture contained 0.8 to 1.1 nkat of chitobiosidase activity/mg, and 154 to 165 units of endochitinase activity/mg. Dietary supplementation with the mixture of enzymes resulted in significant reductions in larval weight (expressed as percentage of the control) ($F=121.56$, $df=3,23$, $P=0.0001$), % pupation ($F=34.69$, $df=3,20$, $P=0.0001$), and % adult emergence ($F=6.64$, $df=3,8$, $P=0.015$) for Trichoplusia ni (FIG. 11).

In addition, ingestion of the mixture of chitinolytic enzymes resulted in a dose-dependent increase in mortality for Myzus persicae at 24 and 48 hrs. ($F=20.33$ and 20.02, respectively; $df=5,15$, $P=0.0001$), Bemisia argentifolii at 18 and 42 hr. ($F=11.23$ and 25.12, respectively; $df=5,11$,P 0.0005) and Hypothenemus hampei ($F=5.84$, $df=3,8$, $P=0.021$) at 30d (FIG. 12).

Separation of endochitinases from chitobiosidases resulted in significantly different levels of mortality for adult B. argentifolii ($F=17.77$, $df=5,19$, $P=0.0001$). Fractions containing endochitinase (peaks I and II) resulted in 80 and 50%O mortality, respectively, after 36 hr. of exposure. The fraction containing both proteins with chitobiosidase activity (peak IV) significantly elevated mortality to 38%, whereas mortality following exposure to a single (most acidic) chitobiosidase (peak V) did not differ from the untreated controls (Peak VI and C) (FIG. 13).

The current study demonstrated that ingestion of an artificial diet containing a mixture of endochitinases and chitobiosidases will significantly reduce the growth and development of larval Trichoplusia ni (FIG. 11) and significantly decrease the survival of Myzus persicae, Bemisia argentifolii, and Hypothenemus hampei (FIG. 12). Separation of the endochitinases from the chitobiosidases was accomplished by perfusion chromatography (FIGS. 9 and 10). Feeding studies indicated that the endochitinases dramatically reduced survival of Bemisia argentifolii (which may have been due to acute toxicity or repellency following ingestion), while the chitobiosidases had less of an effect on survival. These results are in contrast to previous work that demonstrated that the chitobiosidases from Streptomyces albidoflavus significantly reduced the growth and survival of the plant pathogenic fungi Botrytis cinerea and Fusarium oxysporum (Broadway, et al., "Partial Characterization of Chitinolytic Enzymes from Streptomyces albidoflavus," Lett. Appl. Microbiol., 20:271-76 (1995), which is hereby incorporated by reference).

The results of this study provided fundamental information required for evaluating the impact of chitinolytic enzymes on the growth and/or development of herbivorous insects.

Example 14

Biological Activity of Chitinolytic Enzymes against Fungi

Bioassays (following the methods of Lorito, M., Harman, G. E., Hayes, C. K., Broadway, R. M., Tronsmo, A., Woo, S. L. and DiPietro, A., "Chitinolytic Enzymes Produced by Trichoderma harzianum: Antifungal Activity of Purified Endochitinase and Chitobiase," Phytopathology 83:302-07 (1993), which is hereby incorporated by reference) indicated that the purified chitobiosidases significantly reduced spore germination and germ tube elongation for Botrytis cinerea (ED.sub.50 =16 .mu.g/ml) and Fusarium oxysporum (ED.sub.50 =18-26 .mu.g/ml) (See FIG. 14).

Example 15

Isolation of Genes that Encode Chitinolytic Enzymes from S. albidoflavus

The amino-terminal sequence of a chitobiosidase (i.e., with a molecular mass of 31 kD and isoelectric point of 3.9) and an endochitinase (i.e., with an isoelectric point of 6.3 and molecular mass of 41.6 kD) was determined at the Analytical Chemistry Facility, Cornell Biotechnology Program. Degenerate oligonucleotides were synthesized at the Oligonucleotide Synthesis Facility, Cornell Biotechnology Program and at the Entomology Department, using departmental facilities, based on the N-terminal sequences of the endochitinase and chitobiosidase proteins. Additional oligonucleotides were synthesized based on endochitinase homology region 2 (Hayes, et al., "Isolation and sequence of an endochitinase gene from a cDNA library of Trichoderma harzianum," Gene, 135:143-148 (1994), which is hereby incorporated by reference), and a conserved region found from a comparison of exochitinases from S. lividans and S. olivaceoviridis.

Genomic DNA was extracted from an overnight culture of bacteria grown in trypticase soy broth using standard techniques (Hopwood, et al., Genetic Manipulation of Streptomyces: A Laboratory Manual, John Innes Foundation, Norwich (1985), which is hereby incorporated by reference). The DNA was partially digested with Sau3AI, ligated to LambdaGem-11 BamHI arms (Promega), and packaged with Gigapack II extract (Stratagene).

Oligonucleotides specific to the endochitinase (N-terminal and homology region 2 oligonucleotides) were used in PCR to amplify a portion of the endochitinase gene from bacterial genomic DNA. A prominent band of approximately 400 base pairs was detected after gel electrophoresis of PCR products. This band was electroeluted and sequenced directly using dye labelled terminators, thermal cycling, and an ABI automatic sequencer according to the manufacturer's instructions. A peptide sequence derived from this DNA from S. albidoflavus had 90% identity with an endochitinase protein sequence from S. plicatus (Robbins, et al., "Cloning and High-Level Expression of Chitinase-Encoding Gene of Streptomyces plicatus," Gene, 111:69-76 (1992), which is hereby incorporated by reference), indicating that a fragment of an endochitinase gene from S. albidoflavus was isolated. This fragment was used to screen the genomic library. Several clones were plaque-purified and all were found to contain the same hybridizing SalI fragments. The 1,500 bp of a phage clone were sequenced. This region contained a potential open reading frame of 311 amino acids which had 79% identity to the endochitinase of S. plicatus. Each gene was subcloned and their individual sequences were determined.

The chitobiosidase-specific oligonucleotides (N-terminal and exochitinase homology) were used in PCR to amplify a portion of the chitobiosidase gene. A fragment of about 220 bp was electroeluted, sequenced, and used to screen the genomic library as described above. A 4.5 kbp NotI-XhoI fragment was subcloned from a plaque-purified phage clone into pBS and sequenced. This fragment contained a potential open reading frame with a peptide sequence of 74% identity to an exochitinase from S. lividans.

Bacterial genomic DNA was digested with one of several enzymes and analyzed by genomic Southern blotting. Several hybridizing bands in each restriction digest were present on blots probed at reduced stringency with the endochitinase PCR fragment. The endochitinases of S. albidoflavus are believed to be members of a small family of 3-4 genes. At least two of these endochitinase genes have been cloned.

On blots probed at reduced stringency with the chitobiosidase PCR product, a unique band was seen in all digests. This suggests that the cloned chitobiosidase has a unique gene. As reported above, two distinct chitobiosidase proteins were seen on protein gels. Two proteins could be produced from the same gene by post-translational modification of a single peptide, or the two proteins and their genes may be closely related. The N-terminal sequence of the second chitobiosidase protein was determined. The sequence obtained was distinct from the cloned chitobiosidase.

The cloned chitinase genes were expressed in E. coli to obtain pure, individual proteins to produce antibodies against chitinases. Chitobiosidase expression in E. coli was obtained using the pTrcHis vector (Invitrogen). PCR was used to amplify the chitobiosidase gene and to introduce appropriate flanking restriction sites. Features of the pTrcHis expression system are (1) IPTG-inducible, high level expression, (2) a strong repressor, and (3) the recombinant protein is expressed as a fusion with 6 histidine residues and a short linker. The 6.times.His residues allow for rapid affinity selection on nickel containing resins (Ni-NTA, Quagen) with mild elution conditions (low pH or imidazole competition) and the linker contains an enterokinase cleavage site to remove the 6.times.His tail.

The chitobiosidase gene was expressed at high levels in E. coli. Induced, overnight cultures contained equivalent chitobiosidase activity as 5 day chitin-induced cultures of S. albidoflavus. Recombinant chitobiosidase binds strongly to the Ni-NTA resin and is effectively eluted at a pH of 5.5.

The same protocol was followed to produce recombinant endochitinases.

Example 16

Transgenic Tomato Plants

Three binary plasmids were constructed, and Agrobacterium-mediated plant transformation was chosen to carry out tomato and apple transformation. Target genes were cloned into binary plasmid under the control of appropriate promotors. The following plasmids were constructed for this purpose:

Biase19: One S. albidoflavus chitobiosidase gene was amplified by PCR and put under control of a double CaMV 35S promoter and a nopoline synthase terminator (i.e. NPTII) in the expression vector pBin19.

Endo19: One S. albidoflavus endochitinase gene was amplified by PCR and put under the control of a double CaMV 35S promoter and a nopoline synthase terminator in the expression vector pBin19.

BiaseEndo19: The chitobiosidase gene and the endochitinase gene are both put under the control of a double CaMV 35S promoter and a nopoline synthase terminator, respectively, then tandemly ligated in the expression vector pBin19.

Agrobacterium tumefaciens strain LBA4404 was successfully transformed with all three constructs using electroporation.

Four tomato strains were used for transformation. A processing tomato (Lycopersicon esculentum, UC82B) was selected for initial transformation, because it is commercially used by the tomato industry. However, UC82B is a slow growing variety, significantly prolonging the transformation process. Two fast growing strains (Better Boy VFN and Beefmaster VFN) were later transformed. In addition, another strain, Geneva80 was also transformed, because it was locally developed and successfully transformed with other genes.

All three constructs were used to transform the plants. However, emphasis was placed on the double construct BiaseEndo19, and most of the transgenic plants were generated using this construct. Transformants included:

53 transgenic UC82B tomato plants from 27 lines;

15 transgenic Beefmaster tomato plants from 11 lines;

3 transgenic Better Boy tomato plants from 3 lines; and

11 transgenic Geneva80 tomato plants from 8 lines.

Antibodies were raised against both the chitobiosidases and endochitinases.

ELISA assays were used to test the expression of NTPII gene, the Biase gene, and the Endo gene. At least 20 UC82B plants, 9 Beefmaster plants, and 1 Better Boy plant are expressing high level of NPTII: 25 UC82B plants, 12 Beefmaster plants, 2 Better Boy plants, and 5 Geneva80 plants are expressing the Biase gene. The expression level of Biase is low in most of the plants, but at least 4 plants (UC.BE2.A1, UC.BE3.A8, UCBE4.H3, BM.BE1.K1) are expressing a relatively high level of Biase.

Chitin is a target site that distinguishes arthropods from higher organisms. Chitin is a principle component of the insect's exoskeleton (which includes the foregut and hindgut) and peritrophic membrane (which surrounds the midgut), and is essential for structural integrity of insects. Digestion of chitin located in the digestive tract (i.e., foregut, hindgut, and/or peritrophic membrane) may significantly disrupt nutrient acquisition, digestion, and/or absorption, along with other protective and/or physiological functions. For example, the peritrophic membrane is thought to function as (1) protection against abrasion of the gut wall (the site of absorption and some digestion), (2) compartmentalization of digestion in the midgut lumen, and (3) a barrier to pathogens and phytotoxins. Destruction of the peritrophic membrane should have a negative impact on all three of these functions that are essential for the survival of insects. Ingestion of an artificial diet containing a mixture of chitinolytic enzymes will significantly reduce the growth and/or development of Trichoplusia ni, and survival of Myzus persicae, Hypothenemus hampei, and Bemisia tabaci. In addition, chitinolytic enzymes function within the midgut fluid and will digest the peritrophic membrane, in vitro.

Plants are known to contain chitinolytic enzymes that function as an effective phytochemical defense against plant pathogens. There are no reports in the literature to indicate that researchers have examined the effect of plant chitinolytic enzymes on herbivorous insects. However, plant chitinolytic enzymes may have no biological activity against herbivorous insects due to the pH requirements of these enzymes. Plant chitinolytic enzymes, in general, require an acidic environment for activity. They have little or no activity in an alkaline environment. However, herbivorous insects, in general,

have alkaline midguts (Berenbaum, M., "Adaptive Significance of Midgut pH in Larval Lepidoptera," Amer. Natural., 115:138-46 (1980); Grayson, J. M., "Acidity-Alkalinity in the Alimentary Canal of Twenty Insect Species," Virginia J. Sci., Jan:46-59 (1951); Mishra, et al., "pH Trends in the Gut of Xylophagous Insects and Their Adaptive Significance," Mater. Organ., 22:311-19 (1987), which are hereby incorporated by reference). Therefore, chitinolytic enzymes from plants probably have no activity within the lumen of the insect gut. Another source of chitinolytic enzymes is insects which use the enzymes to digest chitin during the moulting process. However, insects also produce factors that will regulate the production and activity of these enzymes, which may interfere with use of these proteins. Therefore, bacteria or other microbes may be the most appropriate source of chitinolytic enzymes for use against insects.

Example 17

Transformation of Tomato or Apple Plants with Gene for Chitinolytic Enzymes Enhances Resistance to Insects

Bioassays were conducted according to the procedures of Broadway, et al., "Influence of Cabbage Proteinase Inhibitors in situ on the Growth of Larval Trichoplusia and Pieris rapae," J. Chem. Ecol., 18:1009-024 (1992), which is hereby incorporated by reference. The following results were achieved.

Larval Heliocoverpa virescens are significantly smaller when they feed on Macintosh apple plantlets that were transformed with the gene for chitobiosidase than when they feed on non-transformed (control) Macintosh plantlets [average larval weight: 289 mg (transformed plants) vs 385 mg (control plants)].

Larval Trichoplusia ni are significantly smaller when they feed on Beefmaster tomato that has been transformed with the chitobiosidase+endochitinase genes than when they feed on non-transformed (control) Beefmaster tomato [average larval weight: 30 mg (transformed plants) vs 143 mg (control plants)].

Tomato plants that were transformed with the chitobiosidase+endochitinase genes showed significantly less feeding damage by larval Trichoplusia ni than control plants [relative level of feeding damage: 2% (transformed plants) vs 45% (control plants)].

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

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             (C) STRANDEDNESS:
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US PATENT & TRADEMARK OFFICE

PATENT FULL TEXT AND IMAGE DATABASE

United States Patent **6,303,844**
Morita , et al. **October 16, 2001**

Method of decontaminating medium containing polychlorinated biphenyls or dioxins

Abstract

A method of decontaminating a medium containing polychlorinated biphenyls (PCBs) or dioxins is disclosed. The contaminated medium is brought into contact with hairy roots derived through introduction of an Ri plasmid from Agrobacterium rhizogenes into a plant capable of absorbing, or absorbing and decomposing PCBs or dioxins, or is brought into contact with regenerated plantlets derived from the hairy roots, to thereby cause the hairy roots or regenerated plantlets to absorb, or absorb and decompose PCBs or dioxins.

Inventors: **Morita; Masatoshi** (Tsukuba, JP); **Yamazaki; Takeshi** (Sakura, JP); **Kamiya; Takashi** (Sakura, JP); **Takano; Hiroyuki** (Sakura, JP); **Fuse; Osamu** (Sakura, JP); **Manabe; Eichi** (Sakura, JP); **Maruta; Toshihisa** (Sakura, JP)

Assignee: **Taiheiyo Cement Corporation** (Tokyo, JP)

Appl. No.: **456297**

Filed: **December 8, 1999**

Foreign Application Priority Data

Dec 09, 1998[JP]	10-350053
Dec 11, 1998[JP]	10-353652

Current U.S. Class: **588/207**; 210/602
Intern'l Class: A62D 003/00; C02F 003/32
Field of Search: 588/205,206,207 75/711,712 210/602

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Primary Examiner: Griffin; Steven P.
Assistant Examiner: Nave; Eileen E.
Attorney, Agent or Firm: Oblon, Spivak, McClelland, Maier & Neustadt, P.C.

Claims

What is claimed is:

1. A method of decontaminating a medium containing polychlorinated biphenyls (PCBs) or dioxins with a plant, which comprises:

a) adding a nonionic surfactant or dimethyl sulfoxide or a combination thereof to the medium containing the PCBs or dioxins or both; and thereafter

b) bringing the medium containing the PCBs or dioxins or both in contact with hairy roots obtained from introduction of an Ri plasmid from Agrobacterium rhizogenes into the plant which absorbs, or absorbs and decomposes the PCBs or dioxins or both;

wherein the dimethyl sulfoxide or the nonionic surfactant or the combination thereof is added to the medium in an amount of 0.005 to 1.0% by weight; and

wherein the plant belongs to a genus selected from the group consisting of ***Solanaceae,*** Cruciferae,

Umbelliferae, Clenopodiaceae, Leguminosae, Compositae and Saxifragaceae.

2. The method of claim 1, wherein said dimethyl sulfoxide is added to the medium.

3. The method of claim 1, wherein said nonionic surfactant is added to the medium.

4. The method of claim 1, wherein a combination of said dimethyl sulfoxide and the said nonionic surfactant is added to the medium.

5. The method of claim 1, wherein the nonionic surfactant is selected from the group consisting of glycerin fatty acid esters, propylene glycol fatty acid esters, sorbitan fatty acid esters, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene sorbitan tetraoleate, polyoxyethylene alkyl ethers, polyoxyethylene polyoxypropylene glycol, polyoxyethylene polyoxypropylene alkyl ethers, polyethylene glycol fatty acid esters, castor oil-modified polyoxyethylene, hardened castor oil-modified polyoxyethylene, polyglycerin fatty acid esters and alkyl glycosides.

6. The method of claim 1, wherein the plant is Atropa belladonna.

7. The method of claim 1, wherein the plant is Brassica juncea var. multiceps.

8. The method of claim 1, wherein the plant is Brassica junceca var. cernua.

9. The method of claim 1, wherein the plant is Brassica juncea var. rapa.

10. The method of claim 1, wherein the plant is Daucus caroia.

11. The method of claim 1, wherein the amount of nonionic surfactant or dimethyl sulfoxide or combination thereof is from 0.01 to 0.1% by weight.

12. The method of claim 1, wherein the medium is soil.

13. The method of claim 1, wherein the medium is a lake.

14. The method of claim 1, wherein the medium is a marsh.

15. A method of decontaminating a medium containing polychlorinated biphenyls (PCB) or dioxins with a regenerated plantlet, comprising:

a) adding a nonionic surfactant or dimethyl sulfoxide or a combination thereof to the medium containing the PCBs or dioxins or both; and thereafter

b) bringing the medium containing the PCBs or dioxins or both into contact with the regenerated plantlet obtained from hairy roots which are obtained from the introduction of Ri plasmid to Agrobacterilim rhizogenes to thereby cause the regenerated plantlet to absorb, or absorb and decompose the PCBs or dioxins or both;

and wherein the dimethyl sulfoxide or the nonionic surfactant or the combination thereof is added to the medium in an amount of 0.01 to 0.1% by weight; and

wherein the regenerated plantlet belongs to a genus selected from the group consisting of ***Solanaceae,***

Cruciferae, Umbelliferae, Chenopodiaceae, Leguminosae, Compositae and Saxifragaceae.

16. The method of claim 15, wherein said dimethyl sulfoxide is added to the medium.

17. The method of claim 15, wherein said nonionic surfactant is added to the medium.

18. The method of claim 15, wherein a combination of said dimethyl sulfoxide and said nonionic surfactant is added to the medium.

19. The method of claim 17, wherein the nonionic surfactant is selected from the group consisting of glycerin fatty acid esters, propylene glycol fatty acid esters, sorbitan fatty acid esters, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene sorbitan tetraoleate, polyoxyethylene alkyl ethers, polyoxyethylene polyoxypropylene glycol, polyoxyethylene polyoxypropylene alkyl ethers, polyethylene glycol fatty acid esters, castor oil-modified polyoxyethylene, hardened castor oil-modified polyoxyethylene, polyglycerin fatty acid esters and alklyl glycosides.

20. The method of claim 15, wherein the plantlet is Atropa belladotilia.

21. The method of claim 15, wherein the plantlet is Brassica juncea var. multiceps.

22. The method of claim 15, wherein the plantlet is Brassica juncea var. cernua.

23. The method of claim 15, wherein the plantlet is Brassica juncea var. rapa.

24. The method of claim 15, wherein the plantlet is Daucus carota.

25. The method of claim 15, wherein the medium is soil.

26. The method of claim 15, wherein the medium is a lake.

27. The method of claim 15, wherein the medium is a marsh.

Description

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a method of efficiently decontaminating a medium which contains polychiloinated biphenyls (hereinafter abbreviated as PCBs) or polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (hereinafter abbreviated as dioxins), such as soil or a lake or marsh contaminated with PCBs or dioxins, through utilization of plants' capability of absorbing, or absorbing and decomposing, PCBs or dioxins.

2. Description of the Related Art

Since PCBs are chemically very stable and exhibit excellent electrical insulating properties, they have been widely used in, for example, insulating oils of capacitors or transformers, thermal media, machine oils, and pressure-sensitive paper.

Since recognition of biological toxicity of PCBs (for example, in the Kanemi Oil case in Japan), production of PCBs has been regulated. However, contamination with PCBs has spread globally, and the effect of PCBs has lasted over a long period of time.

Recent reports have pointed out the toxicity of PCBs as endocrine disrupters and that even a very small amount of PCBs has an adverse effect on the environment.

PCB decomposition processes approved in Japan under the Waste Disposal and Public Cleansing Law are an incineration process, a base-catalyzed decomposition (BCD) process, a chemical extraction-decomposition process (DMI/NaOH process), a potassium tertiary butoxide process (t-BuOK process), a catalitic hydrogenation-dechlorination process (+t-BuOK process), and a supercritical water oxidation process (SCWO process).

Such chemical or physical decomposition processes exhibit relatively high decomposition efficiency, but conventionally involve high processing cost. Also, these processes are suited for decontaminating a contaminated medium of high contamination concentration, but are not suited for practical decontaminating of a large-scale, contaminated medium of low contamination concentration.

Meanwhile, dioxins, which are very toxic, are chemically relatively stable and thus are not easily decomposed. Since dioxins are soluble in oils and fats, once food contaminated with dioxins is taken into the human body, dioxins are absorbed by the organs. Since ingested dioxins are hardly egested, dioxins reportedly not only keep accumulating within the human body but are also transmitted from a mother to a fetus via the womb.

In recent years, as the action of dioxins as endocrine disrupters has become clear, the effect of dioxins on future generations has become of serious concern.

Dioxins are generated mainly in an incineration process at a temperature of about 300-850.degree. C., as in a refuse incineration furnace, and are released into the atmosphere in the form of exhaust gas. Thus-released dioxins are primarily accumulated in, for example, soil, lakes and marshes, and rivers. Since measures against dioxin sources have begun to work, fresh generation of dioxins tends to be suppressed. However, dioxins in the soil have been left accumulated over a long period of time without deterioration or decomposition, so that the soil contaminated with dioxins has become a secondary source of dioxins.

Incineration at high temperature is the most effective process for decomposing dioxins. This incineration process is not suited for treating a large amount of contaminated media and involves considerable treatment. According to a conventional method for disposing of, for example, contaminated soil, the contaminated soil is excavated and relocated to a disposal yard, where the contaminated soil is filled into an excavated hole and covered with sealing soil. This method is conventionally employed, since a relatively large amount of contaminated media can be disposed of.

This filling-up method is a tentative one--a medium contaminated with dioxins is merely moved from one location to another. The relocated, contaminated medium may raise another environmental contamination problem in the new location. Efficient, perpetual measures to treat dioxins have not been achieved.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method of efficiently decontaminating soil or a lake or marsh contaminated with PCBs or dioxins; particularly, a medium contaminated widely and at low concentration with PCBs or dioxins, through utilization of plants' capability of absorbing, or absorbing and decomposing PCBs or dioxins.

The present inventors studied hairy roots derived through infection with Agrobacterium rhizogenes (hairy-root disease bacteria), or soil bacteria. The hairy roots are derived through incorporation into a plant-cell chromosome of the T-DNA region of the Ri plasmid present in the body cell of Agrobacterium rhizogenes followed by development thereof. As compared to common roots, which cannot survive unless special measures are taken, the hairy roots can survive in a common sterile medium and grow abundantly. Even when Agrobacterium rhizogenes is completely sterilized by means or, for example, antibiotics, the hairy roots grow abundantly without losing their properties. The hairy roots can regenerate into plantlets under appropriate culture conditions. The regenerated plantlets are characterized by development of a root system.

The present inventors reached the inventive concept of decontaminating soil or a lake or marsh contaminated with PCBs or dioxins through utilization of the abundantly growing hairy roots or regenerated plantlets derived from the hairy roots and through reinforcement of their inherent capability of absorbing, or absorbing and decomposing PCBs or dioxins. On the basis of this idea, the inventors conducted various experiments and achieved the present- invention.

According to a method of the present invention of decontaminating a medium containing PCBs or dioxins, the contaminated medium is brought into contact with hairy roots derived through introduction of an Ri plasmid from Agrobacterium rhizogenes into a plant capable of absorbing, or absorbing and decomposing PCBs or dioxins, or is brought into contact with regenerated plantlets derived from the hairy roots, to thereby cause the hairy roots or regenerated plantlets to absorb, or absorb and decompose PCBs or dioxins.

The decontaminating method of the present invention not only is efficient but also enables low-cost treatment, plain treatment not involving excavation, on-site treatment not involving relocation of contaminated soil, and perpetual treatment capable of extinguishing PCBs or dioxins. Planting prevents diffusion of soil contaminated with PCBs or dioxins which would otherwise take place through outflow or scattering of surface soil; i.e., contaminants can be decomposed or cleaned up while revegetation is carried out. Even when PCBs or dioxins are not satisfactorily decomposed, undecomposed PCBs or dioxins are absorbed into plants to be condensed and fixed therein, thereby preventing diffusion of contamination. The plants may be reaped and incinerated.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As used herein, the expression "medium containing PCEs or dioxins" refers to a contaminated medium, such as soil, a lake or marsh, or a river contaminated with PCBs or dioxins.

A plant used in the present invention is not particularly limited so long as it has the capability of absorbing, or absorbing and decomposing PCBs or dioxins. Examples of planes capable of absorbing, or absorbing and decomposing PCBs include plants belonging to Solanaceae, Cruciferaa, or Umbelliferae; specifically, Atropa belladonna, Wasabia japonica, and Armoracia rusticana. Particularly, Atropa belladonna is preferred. Examples of plants capable of absorbing, or absorbing and decomposing dioxins include plants belonging to Solanaceae, Cruciferae, Umbelliferae, Chenopodiaceae, Leguminiosae, Compositae, or Saxifragaceae. Among those plants, those belonging to Cruciferae are preferred. Atropa belladonna is particularly preferred. These plantlets may be used

in their original form. Alternatively, a portion of a plantlet, such as seeds, hairy roots, root tissue, or cells, may be used.

Agrobacterium rhizogenes used in the present invention for introduction of an Ri plasmid into a plant is not particularly limited. Agrobacterium rhizogenes is available from, for example, Institute for Fermentation, Osaka.

A method of introducing an Ri plasmid into a plant is not particularly limited. Examples of the method include direct inoculation of Agrobacterium rhizogenes, electrical simulation, and a leaf disk process, which may be selected as appropriate.

A plant into which an Ri plasmid is introduced is cultivated in an appropriate culture medium so as to derive hairy roots. The hairy roots which have undergone bacterial elimination proliferate actively even in a culture medium that does not contain plant hormones, enabling infinite proliferation. Also, the hairy roots can be regenerated into plantlets under appropriate culture conditions.

A medium containing PCBs or dioxins may be brought into contact with the above-mentioned hairy roots or regenerated plantlets derived from the hairy roots, by appropriate means. For example, the hairy roots or regenerated plantlets may be planted or immersed in soil or water contaminated with PCBs For dioxins. The hairy roots or regenerated plantlets absorb, or absorb and decompose PCBs or dioxins contained in the medium, thereby reducing the concentration of PCBs or dioxins contained in the contaminated soil or water.

Preferably, dimethyl sulfoxide and/or a surfactant is added to the medium containing PCBs or dioxins before the medium is brought into contact with the hairy roots or regenerated plantlets. Such an additive accelerates absorption, or absorption and decomposition of PCBs or dioxins effected by the hairy roots or regenerated plantlets. Such a surfactant is not particularly limited, but may be nonionic, anionic, cationic, amphoteric, natural, or synthetic. A surfactant that less affects the growth of the hairy roots or regenerated plants is preferred.

Examples of nonionic surfactants include glycerin fatty acid esters, propylene glycol fatty acid esters, sorbitan fatty acid esters, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene sorbitol tetraoleate, polyoxyethylene alkyl ethers, polyoxyethylene polyoxypropylene glycol, polyoxyethylene polyoxypropylene alkyl ethers, polyethylene glycol fatty acid esters, castor oil-modified polyoxyethylene, hardened castor oil-modified polyoxyethylene, polyglycerin fatty acid esters, and alkyl glycosides. Examples of anionic surfactants include fatty acid salts, polyoxyalkylene alkyl ether acetate salts, alkylsulfate salts, polyoxyalkylene alkyl ether sulfate salts, sulfosuccinic acid surfactants, sulfosuccinamate surfactants, polyoxyalkylene alkylamide ether sulfate salts, monoglyceride sulfate salts, olefinesulfonate salts, alkanesulfonate salts, acylated isethionate salts, acylated amino acid salts, alkylphosphate salts, and polyoxyalkylene alkyl ether phosphate salts. Examples of cationic surfactants include dialkyldimethylammonium salts, alkyltrimethylammonium salts, alkyldimethylbenzylammonium salts, benzalkonlum salts, benzethonium salts, alkylpyridinium salts, and alkylisoquinolinium salts, and surfactants derived from natural products such as lecithin. Examples of amphoteric surfactants include amidoamino acid surfactants, carbobetaine surfactants, sulfobetain surfactants, amidosulfobetain surfactants, imidazoliniumbetain surfactants, amino acid betaine surfactants, and phosphobetaine surfactants. Specific examples include alkyldiaminoethylglycine salts, .beta.-alkylaminopropionic acid salts, and 2-alkyl-N-carboxymethyl-N-hydroxyethylimidazoliniumbetaine.

Among those surfactants, a nonionic surfactant is particularly preferred. The surfactants may be used

singly or in combination. Dimethyl sulfoxide or a surfactant may be added to the medium containing PCBs or dioxins in an amount of 0.005-1.0% by weight, preferably 0.01-0.1% by weight.

EXAMPLES

The present invention will next be described in detail by way of example, which should not be construed as limiting the invention.

Example 1

A. Derivation of Hairy Roots of Atropa Belladonna

(1) Preparation of Atropa Belladonna Material

Seeds of Atropa belladonna were washed with a neutral detergent and were then rinsed with water. The seeds were immersed for 10 minutes in an aqueous solution of sodium hypochlorite having an effective chlorine concentration of 1% to thereby sterilize seed surface. The seeds were washed twice in sterilized water. Subsequently, the seeds were planted and cultivated in a 1/2 (macro) MS culture medium (Murashige and Skoog's Medium; Murashige T. and Skoog F., A revised medium for rapid growth and bioassays with tabacco tissue cultures., Physiol. Plant. 15: 473-497, 1962).

(2) Inoculation of Agrobacterium Rhizogenes

A seedling (hypocotyl) from sterile seeding was cut to a length of about 3 cm. The thus-cut hypocotyl was stuck into a culture medium such that the root-side end faces up while the leaf-side end is stuck in the medium (upside down). Agrobacterium rhizogenes cultivated in agar was taken by means of a platinum loop and was then applied to the upper cut end of the hypocotyl. Then, the hypocotyl was subjected to cultivation in the dark at a temperature of 25.degree. C. Two weeks later, hairy roots (adventitious roots) differentiated from the cut end.

(3) Elimination of Bacteria from Hairy Roots

Agar was prepared by adding a cephalosporin (Claforan, product of Hoechst Japan Ltd.) in an amount of 500 mg/L to a 1/2 (macro) MS culture medium. The above-obtained hairy roots were placed on the agar, followed by cultivation in the dark at a temperature of 25.degree.C. for the purpose of bacterial elimination. This cultivation was repeated 5 times at intervals of one week, achieving complete bacterial elimination.

(4) Cultivation of Hairy Roots

Bacteria-eliminated hairy roots having a length of about 2 cm, 3 hairy roots per petri dish, were cultured in a 1/2 (macro) MS culture medium. Subculturing was carried out every four weeks.

B. Absorption and Decomposition of PCBs by Hairy Roots of Atropa Belladonna

(1) PSBs Reagent

Through use of KANECHLOR 300 (KC300, product of Kaneka Corporation) as a PCBs reagent, a dimethyl sulfoxide (DMSO) solution having a PCBs concentration of 20,000 mg/L was prepared.

(2) Preliminary Cultivation of Hairy Roots and Subsequent Cultivation in PCBs-added Culture Medium

Graded amounts of a 1/2 (macro) MS culture medium (100 mL) were placed in a 300 mL Erlenmeyer flask. Hairy roots (0.2 g) chopped into pieces having a length of about 2 cm were placed in the flask and were subjected to 2-week preliminary cultivation in the dark at a temperature of 25.degree. C. while being shaken (100 rpm). The DMSO solution having a PCBs concentration of 20,000 mg/L prepared above in (1) was placed in another 300 mL Erlenmeyer flask that contains a 1/2 (macro) MS culture medium (100 mL), such that the PCBs concentration of the medium becomes 10 mg/L. In this PCBs-added culture medium, the hairy roots which had undergone the above preliminary cultivation were planted to prepare two kinds of samples--one underwent another 1-week cultivation and the other underwent another 3-week cultivation.

(3) Analysis for PCBs

After completion of cultivation of the hairy roots in the PCBs-added culture medium, the samples were analyzed by means of GC-MS for the amount of PCBs remaining in the culture medium and the amount of PCBs absorbed and accumulated in the hairy roots. The results are shown in Table 1.

Example 2

Hairy roots of Atropa belladonna obtained in a manner similar to that of Example 1 were subjected to preliminary cultivation under the same conditions as those in Example 1. The DMSO solution having a PCBs concentration of 20,000 mg/L mentioned above in Example 1 was placed in another 300 mL Erlenmeyer flask that contains a 1/2 (macro) MS culture medium (100 mL), such that the PCBs concentration or the medium becomes 100 mg/L. Further, Tween20 (polyoxyethylene sorbitan monolaurate), which is a nonionic surfactant, was added into the flask such that the concentration thereof becomes 0.01% by weight. In this PCBs-added culture medium, the hairy roots which had undergone the above preliminary cultivation were planted to prepare two kinds of samples--one underwent another 1-week cultivation and the other underwent another 3-week cultivation.

After completion of cultivation of the hairy roots in the PCBs-added culture medium, the samples were analyzed by means of GC-MS for the amount of PCBs remaining in the culture medium and the amount of PCBs absorbed and accumulated in the hairy roots. The results are shown in Table 1.

Comparative Example 1

In the pre-culturing step in Example 1, instead of hairy roots of Atropa belladonna (0.2 g), Atropa belladonna was sterilely seeded and was cultivated without inoculation of Agrobacterium rhizogenes. The resulting seedling roos were chopped into pieces having a length of about 2 cm. The chopped roots (0.2 g) were subjected to preliminary cultivation in a manner similar to that of Example 1. The DMSO solution having a PCBs concentration of 20,000 mg/L mentioned above in Example 1 was placed in another 300 mL Erlenmeyer flask that contains a 1/2 (macro) MS culture medium (100 mL), such that the PCBs concentration of the medium becomes 10 mg/L. In this culture medIum, the roots which had undergone the above preliminary cultivation were planted to prepare two kinds of samples--one underwent another 1-week cultivation and the other underwent another 3-week cultivation.

After completion of cultivation of the roots in the PCBs-added culture medium, he samples were analyzed by means of GC-MS for the amount of PCBs remaining in the culture medium and the

amount of PCBs absorbed and accumulated in the roots. The results are shown in Table 1.

TABLE 1

	Weight of roots (g)			Percentage to added PCBs (%) 1-week cultivation		3-week cultivation	
	Initial	1-week culti-vation	3-week culti-vation	PCBs in medium	PCBs in roots	PCBs in medium	PCBs in roots
Example 1	0.20	1.16	1.67	58	40	14	64
Example 2	0.20	0.31	0.78	60	17	19	29
Com. Example 1	0.20	0.28	0.43	81	16	68	25

As seen from Table 1, the hairy roots derived through introduction of an Ri plasmid from Agrobacterium rhizogenes into Atropa belladonna absorb and decompose a considerably large amount of PCBs as compared to the roots of natural Atropa belladonna.

Example 3

Hairy roots were derived from Brassica juncea var. multiceps, Brassica juncea var. cernua, Brassica juncea var. rapa, and Daucus carota according to the method of Example 1.

Graded amounts of a 1/2 (macro) MS culture medium (100 mL) were placed in a 300 mL Erlenmeyer flask. Hairy roots (0.2 g) chopped into pieces having a length of about 2 cm were placed in the flask and were subjected to 2-week preliminary cultivation in the dark at a temperature of 25.degree. C. while being shaken (100 rpm) .The DMSO solution having a PCBs concentration of 20,000 mg/L as prepared in Example 1 was placed in another 300 mL Erlenmeyer flask that contains a 1/2 (macro) MS culture medium (100 mL), such that the PCBs concentration of the medium becomes 10 mg/L. In this PCBs-added culture medium, the hairy roots which had undergone the above preliminary cultivation were planted to prepare the respective kinds of samples. The samples were cultivated for another 5 weeks.

After completion of cultivation of the hairy roots in the PCBs-added culture medium, the samples were analyzed by means of GC-MS for the amount of PCBs remaining in the culture medium and the amount of PCBs absorbed and accumulated in the hairy roots. The results are shown n Table 2.

Example 4

The E)MSO solution having a PCBs concentration of 20,000 mg/L mentioned above in Example 1 was placed in a 300 mL Erlenmeyer flask that contains a 1/2 (macro) MS culture medium (100 mL), such that the PCBs concentration of the medium becomes 10 mg/L. Further, Tween20 (polyoxyethylene sorbitan monolaurate), which is a nonionic surfactant, was added into the flask such that the concentration thereof becomes 0.01% by weight. In this PCBs-added culture medium, the hairy roots which had been prepared in a manner similar to that of Example 3 were planted to prepare the respective kinds of samples. The samples were cultivated for another 5 weeks.

After completion of cultivation of the hairy roots in he PCBs-added culture medium, the samples were analyzed by means of GC-MS for the amount of PCBs remaining in the culture medium and the amount of PCBs absorbed and accumulated in the hairy roots. The results are shown in Table 2.

Comparative Example 2

Brassica juncea var. multiceps, Brassica juncea var. cernua, Brassica juncea var. rapa, and Daucus carota were sterilely seeded and were cultivated without inoculation of Agrobacterium rhizogenes. The resulting corresponding seedling roots were chopped into pieces having a length of about 2 cm. The resultant corresponding chopped roots (0.2 g) were subjected to preliminary cultivation in a manner similar to that of Example 3. The DMSO solution having a PCBs concentration of 20,000 mg/L mentioned above in Example 1 was placed in another 300 mL Erlenmeyer flask that contains a 1/2 (macro) MS culture medium (100 mL), such that the PCBs concentration of the medium becomes 10 mg/L. In this culture medium, the corresponding roots which had undergone the above preliminary cultivation were planted to prepare the respective kinds of samples. The samples were cultivated for another 5 weeks.

After completion of cultivation of the roots in the PCBs-added culture medium, the samples were analyzed by means of GC-MS for the amount of PCBs remaining in the culture medium and the amount of PCBs absorbed and accumulated in the roots. The results are shown in Table 2.

TABLE 2

		Weight of roots (g)		Percentage to added PCBS (%) 5-week cultivation	
		Initial	5-week cultivation	PCBs in medium	PCBs in roots
Example 3	Brassica juncea var. multiceps	0.20	2.67	42.1	57.9
	Brassica juncea var. cernua	0.20	2.43	33.0	67.0
	Brassica juncea var. rapa	0.20	0.92	37.5	62.5
	Daucus carota	0.20	2.88	31.2	68.8
Example 4	Brassica juncea var. multiceps	0.20	3.14	5.1	94.9
	Brassica juncea var. cernua	0.20	2.58	4.2	95.8
	Brassica juncea var. rapa	0.20	0.83	21.3	78.7
	Daucus carota	0.20	2.83	18.8	81.2
Com. Example 2	Brassica juncea var. multiceps	0.20	0.31	83.3	16.7
	Brassica juncea var. cernua	0.20	0.28	78.9	21.1
	Brassica	0.20	0.29	80.2	19.8

juncea var. rapa				
Daucus carota	0.20	0.28	89.6	10.4

As seen from Table 2, the hairy roots derived through introduction of an Ri plasmid from Agrobacterium rhizogenes into the plants belonging to Cruciferae and Umbelliferae absorb and decompose a considerably large amount of PCBs as compared to the roots of the natural counterparts of these plants.

Example 5

The DMSO solution having a PCBs concentration of 20,000 mg/L as prepared in Example 1 was placed in a 500 mL culture bottle of glass that contains a 1/2 (macro) MS culture medium (50 mL), such that the PCBs concentration of the medium becomes 10 mg/L. In this PCBs-added culture medium, plantlets of Atropa belladonna regenerated from hairy roots were planted and cultivated at a temperature of 25.degree. C. for 3 weeks under illumination for 16 hours and dark for 8 hours. After completion of cultivation, the regenerated plantlets were analyzed by means of GC-MS for the amount of PCBs remaining in the culture medium and the amount of PCBs absorbed and accumulated therein. The results are shown in Table 3.

Example 6

The DMSO solution having a PCBs concentration of 20,000 mg/L as prepared in Example 1 was placed in a 500 mL culture bottle of glass that contains a 1/2 (macro) MS culture medium (50 mL), such that the PCBs concentration of the medium becomes 10 mg/L. Further, Tween20 (polyoxyethylene sorbitan monolaurate), which is a nonionic surfactant, was added into the bottle such that the concentration thereof becomes 0.01% by weight. In this PCBs-added culture medium, the plantlets of Atropa belladonna regenerated from hairy roots were planted and cultivated for 3 weeks. After completion of cultivation, the regenerated plantlets were analyzed by means of GC-MS for the amount of PCBs remaining in the culture medium and the amount of PCBs absorbed and accumulated therein. The results are shown in Table 3.

Comparative Example 3

Natural Atropa belladonna was planted and cultivated for 3 weeks in a manner similar to that of Example 5. After completion of cultivation, the plantlets were analyzed by means of GC-MS for the amount of PCBs remaining in the culture medium and the amount of PCBs absorbed and accumulated therein. The results are shown in Table 3.

TABLE 3

	Weight of plant bodies (g)		Percentage to added PCBs (%)	
		3-week	3-week cultivation	
	Initial	culti-vation	PCBs in medium	PCBs in plant bodies
Example 5	2.09	2.95	3.9	50.0
Example 6	2.05	3.04	2.2	46.7
Com. Example 3	2.10	2.50	10.0	70.3

As seen from Table 3, the plant bodies of Atropa belladonna regenerated from hairy roots absorb and decompose a considerably large amount of PCBs as compared to those of natural Atropa belladonna.

Example 7

Absorption and Decomposition of Dioxins by Hairy Roots of Atropa Belladonna

(1) Dioxins Reagent

Fly ash in an incineration plant was extracted with toluene. The extract was dissolved in dimethyl sulfoxide (DMSO). The resultant solution was used as a dioxins reagent.

(2) Preliminary Cultivation of Hairy Roots and Subsequent Cultivation in Dioxins-added Culture Medium

Graded amounts of a 1/2 (macro) MS culture medium (100 mL) were placed in a 300 mL Erlenmeyer flask. Hairy roots (0.2 g) chopped into pieces having a length of about 2 cm were placed in the flask and were subjected to 2-week preliminary cultivation in the dark at a temperature of 25.degree. C. while being shaken (100 rpm). The DMSO solution (1 mL) of Idhoxins was placed in another 300 mL Erlenmeyer flask that contains a 1/2 (macro) MS culture medium (100 mL), such that the dioxins concentration of the medium becomes 1000 pg-TEQ/mL. In this dioxins-added culture medium, the hairy roots which had undergone the above preliminary cultivation were planted to prepare two kinds of samples--one underwent another 1-week cultivation and the other underwent another 3-week cultivation.

(3) Analysis for Dioxins

After completion of cultivation of the hairy roots in the dioxins-added culture medium, the samples were analyzed by means of GC-MS for the amount of dioxins remaining in the culture medium and the amount of dioxins absorbed and accumulated in the hairy roots. The results are shown in Table 4.

Example 8

Hairy roots of Atropa belladonna obtained in a manner similar to that of Example 7 were subjected to preliminary cultivation under the same conditions as those in Example 7. The DMSO solution (1 mL) of dioxins mentioned above in Example 7 was placed in another 300 mL Erlenmeyer flask that contains a 1/2 (macro) MS culture medium (100 mL), such that the dioxins concentration of the medium becomes 1000 pg-TEQ/mL. Lurther, Tween20 (polyoxyethylene sorbitan monolaurate), which is a nonionic surfactant, was added into the flask such that the concentration thereof becomes 0.01% by weight. In this dioxins-added culture medium, the hairy roots which had undergone the above preliminary cultivation were planted to prepare two kinds of samples--one underwent another 1-week cultivation and the other underwent another 3-week cultivation.

After completion of cultivation of the hairy roots in the dioxins-added culture medium, the samples were analyzed by means of GC-MS for the amount of dioxins remaining in the culture medium and the amount of dioxins absorbed and accumulated in the hairy roots. The results are shown in Table 4.

Comparative Example 4

Atropa belladonna was sterilely seeded and was cultivated without inoculation of Agrobacterium rhizogenes. The resulting seedling roots were chopped into pieces having a length of about 2 cm. The chopped roots (0.2 g) were subjected to preliminary cultivation in a manner similar to that of Example 7. The DMSO solution (1 mL) of dioxins mentioned above in Example 7 was placed in another 300 mL Erlenmeyer flask that contains a 1/2 (macro) MS culture medium (100 mL), such that the dioxins concentration of the medium becomes 1000 pg-TEQ/mL. In this culture medium, the roots which had undergone the above preliminary cultivation were planted to prepare two kinds of samples--one underwent another 1-week cultivation and the other underwent another 3-week cultivation.

After completion of cultivation of the roots in the dioxins-added culture medium, the samples were analyzed by means of GC-MS for the amount of dioxins remaining in the culture medium and the amount of dioxins absorbed and accumulated in the roots. The results are shown in Table 4.

TABLE 4

	Percentage to added dioxins (%)			
	1-week cultivation		3-week cultivation	
	Dioxins in medium	Dioxins in roots	Dioxins in medium	Dioxins in roots
Example 7	78	13	33	62
Example 8	63	28	26	42
Com. Example 4	78	14	58	27

As seen from Table 4, the above-mentioned hairy roots absorb and decompose a considerably large amount of dioxdins as compared to the roots of natural Atropa belladonna.

Example 9

Granular-red soil (commercially available for gardening use), hard-granular Kanuma soil (commercially available for gardening use), and METROMIX 350 (product of Scotts-Sierra Horticultural Products Company) were mixed in the volume ratios 4:3:3. The resulting mixture was mixed with dry pulverized soil contaminated with high-concentration dioxins, thereby preparing about 1000 pg-TEQ/g of artificial contaminated soil. The artificial contaminated soil (about 2000 g) was placed in a 1/5000a New Wagner Pot (NE-5 type, product of Iuchi Seieido). Atropa belladonna regenerated from hairy roots was planted in the soil. Ninety days later, when roots were found to have sufficiently grown inside the pot, the contaminated soil was analyzed for dioxins concentration. The results are shown in Table 5.

Comparative Example 5

Natural Atropa belladonna was planted in a manner similar to that of Example 9. The contaminated soil was analyzed for dioxins concentration. The results are shown in Table 5.

Comparative Example 6

The contaminated soil of Example 9 in which no plant was planted was allowed to stand for 90 days.

The contaminated soil was analyzed for dioxins concentration. The results are shown in Table 5.

TABLE 5

	Dioxins concentration (pg-TEQ/g)
Example 9	675
Comparative Example 5	814
Comparative Example 6	918

As seen from Table 5, the plant bodies of Atropa belladonna regenerated from hairy roots absorb a considerably large amount of dioxins as compared to those of natural Atropa belladonna.

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PATENT FULL TEXT AND IMAGE DATABASE

United States Patent **6,306,398**

Bathurst , et al. **October 23, 2001**

Compositions which inhibit apoptosis, methods of purifying the compositions and uses thereof

Abstract

The present invention is to methods of obtaining plant-derived compositions that inhibit apoptosis, the compositions obtained thereby, compositions comprising the composition, and methods of use thereof.

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Appl. No.: **862765**

Filed: **May 22, 1997**

Current U.S. Class: **435/325**; 435/360; 435/410

Intern'l Class: A61K 035/78

Field of Search: 424/195.1 435/325,360,410

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Parent Case Text

This is a continuation of U.S. patent application Ser. No. 08/467,035, filed Jun. 6, 1995, U.S. Pat. No. 5,635,187, which is a continuation-in-part of U.S. patent application Ser. No. 08/320,155 filed Oct. 7, 1994, U.S. Pat. No. 5,759,548, which is a continuation-in-part of U.S. patent application Ser. No. 08/158,980, filed Nov. 30, 1993, now abandoned and incorporated herein by reference.

Claims

We claim:

1. A method of obtaining a composition comprising a factor with anti-apoptotic activity wherein the method comprises the steps of

a) delipidating a plant powder by extraction of the powder with a solution containing an organic solvent;

b) separating the powder from the organic solvent;

c) extracting the delipidated powder with an aqueous solution selected from the group consisting of (i) an aqueous solution comprising a water-miscible organic solvent at a concentration of up to 80% of the solution and (ii) phosphate buffered saline; and

d) separating the aqueous solution from the delipidated powder to obtain an aqueous retentate composition comprising a factor with anti-apoptotic activity.

2. The method according to claim 1 wherein the plant powder is delipidated by extraction of the powder with an organic solvent selected from the group consisting of acetone, carbon tetrachloride, ether, hexane and chloroform.

3. The method according to claim 1, wherein the plant powder is obtained from any plant part.

4. The method according to claim 3, wherein the plant part is a storage organ.

5. The method according to claim 4, wherein the storage organ is selected from the group consisting of tubers, seeds and bulbs.

6. The method according to claim 1, wherein the plant powder is derived from the plant families of ***leguminosae,*** solanum and allium.

7. The method according to claim 6, wherein the plant powder is derived from pea or soybean seeds.

8. The method according to claim 6, wherein the plant powder is derived from potato tubers.

9. The method according to claim 1, wherein the aqueous solution is phosphate buffered saline.

10. The method according to claim 1, wherein the water-miscible organic solvents are selected from the group consisting of acetonitrile, lower alkanols, especially C.sub.1 -C.sub.4 alkanols, lower alkanediols, C.sub.2 -C.sub.4 alkanediols, and polymers of lower alkanediols.

11. The method according to claim 10, wherein the water-miscible organic solvent is ethanol.

12. The method according to claim 11, wherein the ethanol is present at a concentration of 50%.

13. The method according to claim 1, further comprising the step of removing the residual water-miscible organic solvent.

14. The method according to claim 13, wherein the step of removing the residual water-miscible organic solvent is by dialysis, ultrafiltration, or lyophilization.

15. The method according to claim 1, further comprising the step of precipitating contaminants of the aqueous retentate.

16. The method according to claim 1, further comprising the step of separating the composition from other components in the aqueous solution by subjecting the aqueous solution to size exclusion gel filtration chromatography.

17. The method according to claim 16, wherein the chromatographic agent employed is selected from the group consisting of Sepharose and BioGel.

18. The method according to claim 17, wherein the chromatographic agent employed is Sepharose S100HR.

19. The method according to claim 16, wherein a chromatography buffer is used and is selected from the group consisting of 0.1 to 0.3 M ammonium bicarbonate or 0.1 to 0.3 M sodium chloride, and 10 to 50 mM phosphate at neutral pH.

20. The method according to claim 19, wherein the chromatography buffer is comprised of 0.1 M ammonium bicarbonate.

21. The method according to claim 16, wherein fractions are collected from the gel filtration chromatography and the fractions with the greatest absorbance at 280 nm are collected and pooled.

22. The method according to claim 16, wherein fractions are collected from the gel filtration chromatography and the fractions with an apparent molecular weight of greater than 80 kD are collected and pooled.

23. The method according to claim 22, wherein fractions are collected from the gel filtration chromatography and those containing the composition are purified and concentrated by dialysis and lyophilization.

24. The method according to claim 23, further comprising the step of extracting the aqueous retentate with a single phase mixture of organic solvents and water to obtain an aqueous phase containing the composition and an organic phase.

25. The method according to claim 24, further comprising the step of lyophilizing the aqueous retentate.

26. The method according to claim 25, further comprising the steps of:

a) extracting the lyophilized material with a single phase mixture of organic solvents and water; and

b) isolating a glycolipid and phospholipid containing fraction by separating the insoluble material from the extracting mixture.

27. The method according to claim 26, wherein the single phase mixture of organic solvents and water comprises chloroform, methanol, and water.

28. The method according to claim 27, wherein the chloroform, methanol and water are present in a ratio of 4:8:3.

29. The method according to claim 26, wherein the separating of step b) is accomplished by a method selected from the group consisting of filtration or centrifugation.

30. The method according to claim 28, further comprising the step of separating by silica chromatography in a mixture of chloroform:methanol.

31. The method according to claim 30 further comprising the step of separating on a diol column.

32. The method according to claim 31 further comprising the step of performing HPLC separation on a silica column to obtain a flow through and five fractions.

33. A method of obtaining a composition with anti-apoptotic activity, comprising the steps of:

a) obtaining a powder of dried peas;

b) delipidating the powder with an organic phase comprising 70% acetone, wherein the organic phase is in a volume approximately equal to the weight of the powder;

c) separating the acetone from the delipidated powder;

d) extracting the delipidated powder with an aqueous phase comprising ethanol and water, wherein the aqueous phase is in a volume approximately equal to the weight of the delipidated powder;

e) separating the aqueous phase from the extracted plant powder to obtain an aqueous retentate;

f) removing the ethanol from the aqueous retentate by filtration; and

g) lyophilizing the product of step f) to obtain a composition with anti-apoptotic activity.

34. The method according to claim 33, further comprising the steps of extracting the lyophilized product of step g) with a single phase mixture of chloroform, methanol, and water present in a ratio of 4:8:3; and isolating a lipid/glycolipid/phospholipid containing fraction separating the insoluble material from the extracting mixture by centrifugation.

35. A method of obtaining a composition comprising a factor with anti-apoptotic activity wherein the method comprises the steps of:

a) delipidating a plant powder by extraction of the powder with a solution containing an organic solvent;

b) separating the powder from the organic solvent;

c) extracting the delipidated powder with an aqueous solution;

d) separating the aqueous solution from the delipidated powder to obtain an aqueous retentate composition comprising a factor with anti-apoptotic activity; and

e) precipitating contaminants of the aqueous retentate composition.

36. A method of obtaining a composition comprising a factor with anti-apoptotic activity wherein the method comprises the steps of:

a) delipidating a plant powder by extraction of the powder with a solution containing an organic solvent;

b) separating the powder from the organic solvent;

c) extracting the delipidated powder with an aqueous solution;

d) subjecting the aqueous solution to size exclusion chromatography to obtain an aqueous retentate composition comprising a factor with anti-apoptotic activity; and

e) precipitating contaminants of the aqueous retentate composite.

Description

FIELD OF THE INVENTION

This invention relates to compositions of matter which are effective in inhibiting apoptotic cell death.

BACKGROUND OF THE INVENTION

Apoptosis is a normal physiologic process that leads to individual cell death. This process of programmed cell death is involved in a variety of normal and pathogenic biological events and can be induced by a number of unrelated stimuli. Changes in the biological regulation of apoptosis also occur during aging and are responsible for many of the conditions and diseases related to aging. Recent studies of apoptosis have implied that a common metabolic pathway leading to cell death may be initiated by a wide variety of signals, including hormones, serum growth factor deprivation, chemotherapeutic agents, ionizing radiation, and infection by human immunodeficiency virus (HIV). Wyllie (1980) Nature 284:555-556; Kanter et al. (1984) Biochem. Biophys. Res. Commun. 118:392-399; Duke and Cohen (1986) Lymphokine Res. 5:289-299; Tomei et al. (1988) Biochem. Biophys. Res. Commun. 155:324-331; and Kruman et al. (1991) J. Cell. Physiol. 148:267-273; Ameisen and Capron (1991) Immunol. Today 12:102-105; and Sheppard and Ascher (1992) J. AIDS 5:143-147. Agents that affect the biological control of apoptosis thus have therapeutic utility in numerous clinical indications.

Apoptotic cell death is characterized by cellular shrinkage, chromatin condensation, cytoplasmic blebbing, increased membrane permeability and internucleosomal DNA cleavage. Gerschenson et al. (1992) FASEB J. 6:2450-2455; and Cohen and Duke (1992) Ann. Rev. Immunol. 10:267-293.

All references cited herein both supra and infra are hereby incorporated herein by reference.

A variety of food supplements containing, in part, partially processed plant extracts have been used to ameliorate the gastrointestinal disorders that often accompany chemotherapy, radiation and AIDS. The supplements generally contain carbohydrates, fat and plant protein hydrolysates. See, e.g., Tomei and Cope et al. in Apoptosis The Molecular Basis of Cell Death (1991) Cold Spring Harbor Laboratory Press.

Several proteinase inhibitors derived from plant extracts have anticarcinogenic activity. Troll et al. (1987) Adv. Cancer Res. 49:265-283. The Bowman-Birk inhibitors are the best described of these inhibitors. Birk (1985) Int. J. Pep. Pro. Res. 25:113-131. Bowman-Birk inhibitors are described as a family of disulfide bonded proteins with a molecular weight of about 8 kD which suppress cellular transformation. Chou et al. (1974) Proc. Natl. Acad. Sci. USA 71:1748-1752; Yavelow et al. (1985) Proc. Natl. Acad. Sci. USA 82:5395-5399; and Yavelow et al. (1983) Cancer Res. (Suppl.) 43:2454s-2459s. Crude soybean extracts containing Bowman-Birk inhibitors have been described. Kennedy et al. U.S. Pat. No. 4,793,996; PCT publication No. WO 94/20121; and Kennedy, A. R. (1994) Cancer Res. (Suppl) 54:1999s-2005s. Bowman-Birk inhibitors have also been described immunologically. WO 90/03574; and U.S. Pat. Nos. 4,959,310; and 5,053,327. Bowman-Birk inhibitors have also been found to have activity in degranulation of macrophages. Japanese Patent No. 63051335.

Lysophosphatidic acid (LPA) is found in a variety of plant products as are a variety of phospholipids. LPA as been found to have a variety of physiological activities including mitogenesis, growth factor and as an anti-wrinkle agent. U.S. Pat. Nos. 4,263,286; 4,746,652; 5,326,690; and 5,340,568. LPA is reviewed in detail by Moolenaar (1994) TICB 4:213-219; and Eichholtz et al. (1990) Biochem. J. 291:677-680.

SUMMARY OF THE INVENTION

The present invention encompasses methods of obtaining compositions that inhibit apoptosis and the compositions obtained thereby. The compositions are termed phytogenic apoptosis inhibitors (PAIs). The invention encompasses physiologically acceptable compositions suitable for administering the PAIs in an amount sufficient to modulate apoptosis. The invention further encompasses methods of use of the PAIs.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 presents the anti-apoptotic effect of PAIs on confluent C3H 10T1/2 cells.

FIG. 2 presents the concentration-dependent anti-apoptotic effect of PAIs on C3H 10T1/2 cells in exponential growth phase.

FIG. 3 presents a comparison of the anti-apoptotic activity of PAIs purified by ethanol extraction and PAIs further purified by size exclusion gel filtration chromatography on C3H 10T1/2 cells. The data are presented as a function of trypsin inhibitory activity.

FIG. 4 presents the anti-apoptotic activity of various concentrations of soy PAIs on quiescent C3H 10T1/2 cells treated with cycloheximide.

FIG. 5 presents a chromatogram of monosaccharides present in PAI.

FIG. 6 depicts the results obtained in the rat cardiac myocytes.

FIG. 7 is a graph depicting the effect of soy flour extracts on methotrexate treated rats.

FIG. 8 is a graph depicting the effect of soy flour extracts on methotrexate treated rats.

FIG. 9 is a graph depicting the effect of soy AcE on methotrexate treated rats.

FIG. 10 is a graph depicting the effect of soy flour extracts on methotrexate treated rats.

FIG. 11 is a bar graph depicting the incidence of rats presenting with diarrhea after treatment with methotrexate and various diets.

FIG. 12 is a bar graph summarizing the incidence of diarrhea and weight gain in rats treated with methotrexate and various diets.

FIG. 13 is a photograph depicting DNA laddering as a measure of apoptosis in lymphocytes obtained from an HIV-infected individual.

FIG. 14 is a bar graph depicting anti-apoptotic activity of lysophosphatidic acid as enhanced by preincubation with BSA or fraction B. The abbreviations used are: LPA, L.alpha.-lysophosphatidic acid, oleoyl (C18:1, [cis]-9); BSA, bovine serum albumIn, fraction V, ethanol extracted; and "B," fraction B from soy flour, predominantly phosphatidyl inositol.

FIG. 15 is a graph depicting the anti-apoptotic effect of lysophosphatidic acid, oleoyl (C18:1, [cis]-9) on serum deprived C3H 10T1/2 cells in vitro.

MODES OF CARRYING OUT THE INVENTION

It has now been found that a variety of plant constituents contain components which, when at least partially purified and purified, exhibit the ability to inhibit apoptosis. These compositions are readily separable from Bowman-Birk inhibitors and are distinct from other, known, therapeutically effective plant products. The composition may vary slightly in chemical constituents depending on the source and growing conditions of the plant from which they are derived. The compositions are referred to herein as "PAIs" as the invention encompasses related compositions made by the methods described herein but obtained from different plant sources. The composition may also be prepared synthetically by methods known in the lipid synthesis art. Several relatively purified compositions are provided and are designated L/G, AcE, MAcE and FAcE, to designate different degrees of purity as discussed below. Relatively pure fractions are also provided designated "D" and "L" as discussed below, each of which is a mixture of phospholipids. Two additional, relatively pure compositions are provided both of which contain lysophosphatidic acid (LPA); LPA and a protein carrier; and LPA and an otherwise inactive fraction "B."

PAIs can be isolated from a variety of different plants and plant organs. Preferably the plants are in the leguminosae (beans and peas etc.) family, but PAIs can be isolated from other plants such as those in the solanum (such as potatoes) and allium (such as garlic) families. PAIs can also be isolated from partially purified plant extracts including, but not limited to, molasses, lecithin, partially purified protein concentrates and partially purified protein hydrolysates. It is within the skill of one in the art, utilizing the methods described herein, to determine whether PAIs can be isolated from a particular species of plant, plant extract or organ within a plant.

Any plant extract or part thereof that yields the compositions is suitable for use in the present invention. The plant organs which can be utilized include, but are not limited to, stems, leaves, roots, flowers, rhizomes, and preferably, storage organs such as seeds, tubers and bulbs. Preferably, the plant part utilized is a storage organ including, but not limited to, potatoes, and garlic. Most preferably the dried seeds of legumes including, but not limited to, soybeans and peas are used for ease of processing. Although the terms "seed" and "seeds" are used herein, it should be understood that these terms encompass any plant part which yields at least one therapeutically active PAI, or PAI that is active in cell culture.

The invention encompasses methods for substantially purifying PAIs. Various degrees of purity can be achieved. The seeds are ground or pulverized, preferably into a powder or flour. As used herein, the term powder refers to a ground dried plant part. The powder particles should be sufficiently small enough to allow substantial surface area exposure to the various liquids to which they are exposed. Any method of grinding or pulverizing is suitable for use herein, typically grinding of seeds is accomplished by a commercial mill. PAIs are unusually heat stable, thus grinding can be done at temperatures that denature many proteins. Seed flours which are purchased commercially can also be used. For instance, soybean flour and various yellow and green pea flours have been found to contain active PAIs.

The seed powder is then delipidated by any method known in the art. It may be necessary to delipidate the powder in an inert environment, for example oxygen-free nitrogen or argon, or to include antioxidants during the procedure, for example BHT or BHA, to improve activity or minimize changes of an oxidative nature. Delipidation is generally accomplished by extracting the powder with a solution containing an organic solvent. Suitable organic solvents include, but are not limited to, acetone, carbon tetrachloride, ether, hexane and chloroform. Typically, the concentration of organic solvent in the solution is from 50-100%. Preferably, the organic solvent is acetone. The concentration of organic solution used may vary with respect to the particular solvent and the seed type; determination of the effective concentration is within the skill of one in the art. Preferably, in the case of acetone, the concentration is about 70%. Multiple organic extractions may also be carried out. Ratios of organic solution to powder (weight/volume) may also vary. Although not limited to the following range, typically the ratios are from about 2:1 to about 1:20 (weight powder/volume organic solution) are employed. In the case of acetone, a ratio of 1:5 is preferred.

Due to the stability of PAIs, the temperature and atmospheric pressure under which delipidation takes place are largely restricted only by the respective freezing and boiling points of the organic solutions employed. Typically, for ease of use, the delipidation takes place at room temperature and atmospheric pressure. The extraction time is likewise not stringent and depends largely upon the ratio of powder to organic solution. In the case of 70% acetone extraction with a ratio of 1:5, delipidation typically takes place for 30 minutes, with constant stirring.

The organic solution is then separated from the powder by any method known in the art. Preferably, the powder is removed from the organic phase by centrifugation and removal of the organic phase. Any all suitable form of separation can also be employed including, but not limited to, filtration or separation by gravity. The PAIs remain in the extracted powder.

The delipidated powder is then extracted with an aqueous solution to yield an aqueous retentate containing the PAIs. The aqueous solution can be a buffered solution such as phosphate buffered saline (PBS) and may also contain up to about 80% water miscible organic solvents. Suitable water miscible organic solvents include, but are not limited to, acetonitrile, lower alkanols, especially C.sub.1 -C.sub.4 alkanols such as ethanol, methanol and propanol, lower alkanediols, especially C.sub.2 -C.sub.4 alkanediols such as ethyleneglycol, and polymers of lower alkanediols, especially polyethyleneglycol. Preferably ethanol is used, most preferably at 50% concentration.

The ratio of aqueous solution to powder can also be varied. The extraction ratio may be from about 1:1 to at least about 1:20 (weight powder/volume aqueous solution). Generally, a 1:10 ratio of 50% ethanol is used. The extraction time varies also and depends on the volume of the aqueous solution and percentage of water miscible organic solvents used. In the case of a 1:10 volume of 50% ethanol, extraction proceeds for 1 hour with constant mixing or stirring (or agitation).

The pH range of the aqueous solution has been found to be largely irrelevant, ranges between 2.5 and 11 have been tested; it is thus likely that an even broader range may be effective. Typically, for ease of use, the pH range is 7-8.

The temperature and atmospheric conditions of the aqueous extraction can vary widely and depend largely on the freezing and boiling points of the aqueous solution. Typically, the extractions are carried out at room temperature and atmospheric pressure. Once the aqueous extraction has taken place, the aqueous retentate is removed for further processing. Any method of removal known in the art is suitable including but not limited to centrifugation and filtration.

The aqueous retentate is then further purified to yield the PAI. Any water-miscible organic solvent is removed by any method known in the art including but not limited to ultrafiltration, drying and dialysis. Ultrafiltration can be performed using a 10 kD molecular weight cut off to remove low molecular weight proteins such as monomers of the Bowman-Birk inhibitors and organic solvents. Likewise the molecular weight cut off of the dialysis tubing can be 10 kD.

Residual organic solvent can be removed by diafiltration after ultrafiltration or by multiple changes of the dialysate, for instance, by pure water. The aqueous retentate can be stored for up to several days in solution and indefinitely as a lyophilized solid. Preferably, the aqueous retentate is lyophilized. The lyophilized solid is ACE if the starting material is soy flour. Both the aqueous retentate and lyophilized retentate may be subject to further processing steps; the lyophilized retentate being resuspended in an appropriate aqueous solution prior to further processing.

Material obtained may be further separated by passage through any molecular weight size exclusion chromatography including, but not limited to, Sepharose S100HR (Pharmacia Biotechnology Piscataway N.J. USA) or BioGel P-100 (BioRad Laboratories Inc, Hercules Calif. USA) in an aqueous buffer. Suitable buffers include, but are not limited to, 0.1 to 0.3 M ammonium bicarbonate or 0.1 to 0.3 M NaCl in 10 to 50 mM phosphate at neutral pH.

The PAIs obtained from size exclusion chromatography are found in the void volume and have an apparent molecular weight of greater than 80 kD. The material found in this fraction may be resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and contains several proteins. Staining with Coomassie Blue indicates the presence of 6-8 proteins of a molecular weight range from 18 to 68 kD. Analysis by thin layer chromatography demonstrates the presence of several lipid-type compounds.

The fractions eluted from the chromatography with the greatest absorbance at 280 nm are coincident with the fractions containing the greatest biological activity. The biological activity separates from low molecular weight material and elutes in the void volume at a position that is consistent with that of a molecular weight of greater than 80 kD or an aggregate. This material may be concentrated, dialyzed, lyophilized and stored indefinitely in a lyophilized form. The lyophilized solid is FAcE, if the starting material is soy flour.

Solubilized PAIs can be precipitated with acetone at a concentration of 70% or more. However, treatment with various agents, including strong acids, destroy their activity. For instance, trifluoroacetic acid, hydrochloric acid, trichloroacetic acid and phenol destroy their activity. However, pH levels as low as 2.5 do not destroy activity, as 1% acetic acid does not affect the activity of PAIs.

The PAIs can be further isolated by extracting a freeze dried high molecular weight fraction obtained from defatted and ethanol extracted seed flour into a single phase mixture of chloroform:methanol:water (4:8:3). This is most conveniently done by dissolving the dried material in the water fraction, then adding methanol followed by chloroform, mixing and removing the pecipitate. This extraction yields a glycolipid/lipid/phospholipid fraction which retains PAI activity.

For example, 0.1 gEQ (the amount derived from 0.1 g of starting material) of the high molecular weight fraction (FAcE) was dissolved in 7.5 ml of water and with constant mixing, 20 ml of methanol was added followed by 10 ml of chloroform. The insoluble material was removed by centrifugation (8,000.times.g.times.10 min) and the PAI reclaimed from the solution by rotary evaporation to remove the organic solvents and freeze-drying to remove the water. The fraction

obtained has been termed the L/G fraction. The carbohydrate composition of the L/G fraction consists of arabinose and galactose in a 3:2 ratio with fucose, rhamnose, glucosamine, glucose and mannose all present as minor constituents.

The L/G fraction can be further separated on the basis of its solubility in a mixture of chloroform:methanol and resolved by silica chromatography either in a chromatography column or by a thin-layer chromatography (TLC) plate format.

The material is then subjected to a preliminary chromatography step on silicic acid, i.e., a Mallinckrodt SiO.sub.2.xH.sub.2 O 100 mesh powder. The active material is loaded in chloroform and washed with chloroform or a chloroform:methanol mixture of 90:10 or 80:20 and eluted with methanol or CHCl.sub.3 :MeOH (10:90 or 20:80).

The active material can be further purified by chromatography on a diol column such as Diol SepPak cartridges (Waters, Millipore). For example, silica purified L/G, crude commercial lecithin, or other soy phospholipid preparations that are soluble in chloroform may be used as a starting material. For example, about 100-1000 mg of the sample is dissolved at a concentration of about 100 mg/ml in chloroform and loaded onto a pre-equilibrated 10 g diol column. The column is washed with 2-5 volumes of chloroform, eluted with 2-5 volumes of isopropanol, eluted with 2-5 volumes of ethanol, and, finally, eluted with 2-5 volumes of methanol. The majority of the activity is eluted in the methanol fraction although some activity is also found in the ethanol fraction. The activity may be isolated from methanol by drying. Suitable methods of drying include, but are not limited to, rotary evaporation or under vacuum. Some samples develop a precipitate upon overnight storage at -20.degree. C; however, this precipitate can be removed by centrifugation without loss of activity.

The active material can be further separated by HPLC chromatography on a silica column such as Dynamax 60A Si column from Rainin Instrument Co., Inc. The gradient used to elute the active material is from 95:3:2 0.05 acetonitrile:methanol:water:ammonium hydroxide to 65:21:14 0.35 acetonitrile:methanol:water:ammonia hydroxide. The elution profile is monitored at 205 nm. As described in the Examples presented below, this stage of purification produces five separate products designated fractions 1-5. These products were isolated and analyzed separately for their composition and anti-apoptotic activity. Several fractions were recombined and assayed for their anti-apoptotic activity. It was found that the flow through contained predominantly lysophosphatidic acid (LPA), which, as described below, is a class of compounds. When assayed for activity, it was found that a commercially available LPA, L-a-lysophosphatidic acid, oleoyl (C18:1, [cis]-9), had no anti-apoptotic activity. LPA found in this fraction, and commercially available LPA in the presence of a protein or proteins to which it specifically binds, do possess anti-apoptotic activity. Thus, one embodiment of the present invention is compositions comprising LPA and an effective amount of a specific binding protein. Preferably, the binding protein is serum albumin. Note that the presence of Bowman-Birk inhibitors does not confer anti-apoptotic activity on LPA. It was also found that LPA in the presence of a fraction designated "B" also possessed anti-apoptotic activity. Fraction "B" primarily phosphatidyl inositol, does not possess anti-apoptotic activity. Thus another embodiment of the invention is a composition comprising LPA and an effective amount of fraction B or an active constituent thereof. Note that fraction B does not contain protein, thus the ability to induce anti-apoptotic activity in LPA is due solely to the presence of phospholipids. While not being bound by any one theory, the effect of fraction B may be due to the formation of a micelle or liposome which protects LPA and/or allows correct presentation of LPA to the cells.

Fraction "D" was also obtained and found to possess anti-apoptotic activity. This fraction corresponds to Peak 3 in Example 8. Thus another embodiment of the invention is compositions comprising

fraction D.

Fraction "L" was also obtained and found to possess anti-apoptotic activity. This fraction corresponds to Peak 5 in Example 8. Thus another embodiment of the invention is compositions comprising fraction L. Fractions D and L may be additive in their anti-apoptotic activity. Thus another embodiment of the invention is compositions comprising fractions D and L.

Thus, the above embodiments are also included in the term PAI and are suitable for use in the indications and compositions described herein. LPA has the structure: ##STR1##

wherein R is an unsubstituted or substituted, saturated or unsaturated, straight or branched chain alkyl having from 11 to about 23 carbon atoms.

Also, as used herein, LPA encompasses a variety of molecules, including, but not limited to, a 2-deoxy- or 2-deoxy-2-halo-lysophosphatidic acid compound having the structure: ##STR2##

or a pharmaceutically-acceptable salt thereof, wherein --R is unsubstituted or substituted, saturated or unsaturated, straight or branched-chain alkyl having from 11 to about 23 carbon atoms; each X is independently O or S; Y is O or CH.sub.2 ; and Z is H, halo, NH.sub.2, SH, OH, or OPO.sub.3 H.sub.2.

Also included is RC(O)O-- being lauryl, myristyl, palmityl, stearyl, palmitoleyl, oleyl or linoleyl; more particularly, oleyl, palmitoleyl, myristyl, palmityl, or lauryl; especially myristyl or lauryl.

Pharmaceutically-acceptable salts of LPAs include, but are not limited to, alkali metal salts, such as sodium and potassium; alkaline earth metal salts, such as calcium and magnesium; non-toxic heavy metal salts; ammonium salts; trialkylammonium salts, such as trimethyl-ammonium and triethylammonium; and alkoxyammonium salts, such as triethanolammonium, tri(2-hydroxyethyl) ammonium, and tromethamine (tris(hydroxymethyl)aminomethane). Particularly preferred are calcium salts.

Preferred compounds useful as LPA in combination with a specific binding protein or fraction B include, but are not limited to, L and D 1-myristoyl-2-fluoro-2-deoxy-glycerol-3-phosphate, L and D 1-lauroyl-2-fluoro-2-deoxy-glycerol-3-phosphate, L and D 1-oleoyl-2-fluoro-2-deoxy-glycerol-3-phosphate, L and D 1-palmitoleoyl-2-fluoro-2-deoxy-glycerol-3-phosphate, L and D myristoyl-2-deoxy-glycerol-3-phosphate, L and D 1-lauroyl-2-chloro-2-deoxy-glycerol-3-phosphate, L and D 1-myristoyl-2-chloro-2-deoxy-glycerol-3-phosphate, and calcium salts thereof.

Other factors which may influence the activity of these compositions are chain length of LPA, presence of cholesterol, presence of micelles, liposomes, detergents, and emulsifying agents, and chain position in LPA, i.e., first or second carbon on the glycerol. In the case of micelles, liposomes and detergents, micelles and liposomes will cause an enhancement of activity whereas detergents or detergent-like molecules will cause a decrease in activity.

The active fractions obtained from HPLC silica chromatography may be further separated on the basis of their hydrophobicity, for instance by HPLC on a C18 column (Dynamax 60A, Rainin Instrument Co. Inc.) in a variety of methanol containing buffers, including, but not limited to, 100% methanol containing 800 mg/l ammonium acetate; 99% methanol containing 1 mM sodium phosphate pH 7.4; and 90% methanol, 10% sodium phosphate pH 7.4. The material is eluted isocratically in 90:10 methanol:5 mM NaPO.sub.4 pH 7.4.

A variety of methods are known in the art for purifying and analyzing lipids. Any method known in the art may be used in the practice of the present invention provided it results in purification of an active fraction. For review, see Bligh and Dyer (1959) Can. J. Biochem. Physiol. 37:911-917; Patton et al. (1982) J. Lipid Res. 23:190-196; Jungalwala (1985) Recent Developments in Techniques for Phospholipid Analysis, in Phospholipids in Nervous Tissues (ed. Eichberg) John Wiley and Sons, pp. 1-44; Hamilton et al. (1992) in the series, A Practical Approach (Richwood et al. eds.) IRL Press at Oxford University Press; and Kates (1986) Techniques of Lipidology: Isolation, Analysis and Identification in Laboratory Techniques in Biochemistry and Molecular Biology (Burdon et al. eds.) Elsevier.

Typically, soy flour, or fractions thereof, is extracted by suspending in water (20% weight per volume in the case of flour) and adding two volumes of methanol and one volume of chloroform. This is a single phase and is stirred/mixed at room temperature for thirty minutes to one hour. To this mixture is added one volume of chloroform, mixed, and one volume of water. This forms two phases and the phases are separated by centrifugation or a separating funnel after first removing any solids (if flour has been used as the starting material). The activity is in the organic (bottom) phase.

The in vitro apoptosis inhibitory activity of the PAIs appears to be largely limited to actively proliferating cells; quiescent cells appear to be relatively unaffected.

The active components of the PAIs are highly stable in the presence of proteases. For instance, the PAIs have been treated with trypsin, chymotrypsin, papain, elastase, subtilisin, and proteinase K under conditions suitable for proteolysis but the proteases have no effect on their activity.

The invention further comprises therapeutic compositions comprising substantially purified PAIs. The level of purity necessary for the composition can be determined empirically and is within the skill of one in the art. The compositions are suitable for use in a variety of disorders, as described below, and in both human and veterinary applications.

The activity of the PAIs, as well as active fractions thereof obtained during the purification method can be measured in any anti-apoptosis assay known in the art. These include, but are not limited to, the serum deprivation of the C3H 10O1/2 cell assay described in detail in commonly owned PCT Publication No. WO 9425621 which is the preferred assay method, as well as the methods described in Examples 3 and 4. Furthermore, in vivo apoptosis inhibition may be measured by any method known in the art.

In general, PAIs are pharmaceutically acceptable due to their low toxicity in the therapeutic dosage range, stability and ability to be incorporated into a wide variety of vehicles for numerous routes of administration. The PAIs can be administered alone or in combination with other pharmaceutically effective agents including, but not limited to, antibiotics, wound healing agents, antioxidants and other therapeutic agents. Suitable antibiotics include, but are not limited to, ampicillin, tetracycline, chloramphenicol and penicillin. Suitable wound healing agents include, but are not limited to, transforming growth factors (TGF-.beta.s), epidermal growth factors (EGFs), fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs). Suitable antioxidants include, but are not limited to, vitamins C and E.

The compositions contain at least a therapeutically effective amount of at least one PAI and may contain at least one physiologically acceptable carrier. A physiologically acceptable carrier is one that does not cause an adverse physical reaction upon administration and one in which PAIs are

sufficiently soluble to deliver a therapeutically effective amount of the compound. The therapeutically effective amount of PAIs depends in part upon the manner of introduction and the indication to be treated and other criteria evident to ordinary skill of one in the art. Typically, a therapeutically effective amount is one sufficient to modulate apoptosis in the condition being treated as evidenced by amelioration of the symptoms. Typically, a therapeutically effective amount is from about 0.5-100% by weight of at least one PAI. The route(s) of administration useful in a particular indication are discussed below and are well known to one of skill in the art.

Routes of administration include, but are not limited to, topical, transdermal, parenteral, gastrointestinal, transbronchial and transalveolar. Topical administration is accomplished via a topically applied cream, gel, rinse, etc. containing therapeutically effective amounts of PAIs. Transdermal administration is accomplished by application of a cream, rinse, gel, etc. capable of allowing the PAIs to penetrate the skin and enter the blood stream. Parenteral routes of administration include, but are not limited to, direct injection such as intravenous, intramuscular, intraperitoneal or subcutaneous injection. Gastrointestinal routes of administration include, but are not limited to, ingestion and rectal. Transbronchial and transalveolar routes of administration include, but are not limited to, inhalation, either via the mouth or intranasally and direct injection into an airway, such as through a tracheotomy.

While the PAIs can be topically administered alone, it may be desirable to administer them in admixture with a topical pharmaceutically or cosmetically acceptable carrier. "Topical pharmaceutically acceptable carrier" as used herein is any substantially non-toxic carrier conventionally usable for topical administration of pharmaceuticals in which the PAIs will remain stable and bioavailable when applied directly to skin or mucosal surfaces. For example, the PAIs can be dissolved in a liquid, dispersed or emulsified in a medium in a conventional manner to form a liquid preparation or mixed with a semi-solid (gel) or solid carrier to form a paste, powder, ointment, cream, lotion or the like.

Suitable topical pharmaceutically acceptable carriers include water, petroleum jelly (vaseline), petrolatum, mineral oil, vegetable oil, animal oil, organic and inorganic waxes, such as microcrystalline, paraffin and ozocerite wax, natural polymers, such as xanthanes, gelatin, cellulose, collagen, starch, or gum arabic, synthetic polymers, such as discussed below, alcohols, polyols, and the like. The carrier may be a water miscible carrier composition that is substantially miscible in water. Such water miscible topical pharmaceutically acceptable carrier composition can include those made with one or more appropriate ingredients set forth above but can also include sustained or delayed release carriers, including water containing, water dispersible or water soluble compositions, such as liposomes, microsponges, microspheres or microcapsules, aqueous base ointments, water-in-oil or oil-in-water emulsions, gels or the like.

In one embodiment of the invention, the topical pharmaceutically acceptable carrier comprises a sustained release or delayed release carrier. The carrier is any material capable of sustained or delayed release of the PAIs to provide a more efficient administration resulting in one or more of less frequent and/or decreased dosage of the PAIs, ease of handling, and extended or delayed effects on dermatologic conditions. The carrier is capable of releasing the PAIs when exposed to any oily, fatty, waxy, or moist environment on the area being treated or by diffusing or by release dependent on the degree of loading of the PAIs to the carrier in order to obtain releases of the PAIs. Non-limiting examples of such carriers include liposomes, microsponges, microspheres, or microcapsules of natural and synthetic polymers and the like. Examples of suitable carriers for sustained or delayed release in a moist environment include gelatin, gum arabic, xanthane polymers; by degree of loading include lignin polymers and the like; by oily, fatty or waxy environment include thermoplastic or

flexible thermoset resin or elastomer including thermoplastic resins such as polyvinyl halides, polyvinyl esters, polyvinylidene halides and halogenated polyolefins, elastomers such as brasiliensis, polydienes, and halogenated natural and synthetic rubbers, and flexible thermoset resins such as polyurethanes, epoxy resins and the like. Preferably, the sustained or delayed release carrier is a liposome, microsponge, microsphere or gel.

The compositions used in the method of treating dermatologic conditions of the invention are applied directly to the areas to be treated. While not required, it is desirable that the topical composition maintain the PAIs at the desired location for about 24 to 48 hours.

If desired, one or more additional ingredients conventionally found in topical pharmaceutical or cosmetic compositions can be included with the carrier: such as a moisturizer, humectants, odor modifier, buffer, pigment, preservative, vitamins such as A, C and E, emulsifier, dispersing agent, wetting agent, odor-modifying agent, gelling agents, stabilizer, propellant, antimicrobial agents, sunscreen, enzymes and the like. Those of skill in the art of topical pharmaceutical formulations can readily select the appropriate specific additional ingredients and amounts thereof. Suitable non-limiting examples of additional ingredients include superoxide dismutase, stearyl alcohol, isopropyl myristate, sorbitan monooleate, polyoxyethylene stearate, propylene glycol, water, alkali or alkaline earth lauryl sulfate, methylparaben, octyl dimethyl-p-amino benzoic acid (Padimate O), uric acid, reticulin, polymucosaccharides, hyaluronic acids, aloe vera, lecithin, polyoxyethylene sorbitan monooleate, vitamin A or C, tocopherol (vitamin E), alpha-hydroxy of alpha-keto acids such as pyruvic, lactic or glycolic acids, or any of the topical ingredients disclosed in U.S. Pat. Nos. 4,340,586, 4,695,590, 4,959,353 or 5,130,298 and 5,140,043.

Because dermatologic conditions to be treated may be visible, the topical carrier can also be a topical cosmetically acceptable carrier. By "topical cosmetically acceptable carrier" as used herein is meant any substantially non-toxic carrier conventionally usable for topical administration of cosmetics in which the PAIs will remain stable and bioavailable when applied directly to the skin surface. Suitable cosmetically acceptable carriers are known to those of skill in the art and include, but are not limited to, cosmetically acceptable liquids, creams, oils, lotions, ointments, gels, or solids, such as conventional cosmetic night creams, foundation creams, suntan lotions, sunscreens, hand lotions, make-up and make-up bases, masks and the like. Thus, to a substantial extent topical cosmetically acceptable carriers and pharmaceutically acceptable carriers are similar, if not often identical, in nature so that most of the earlier discussion on pharmaceutically acceptable carriers also applies to cosmetically acceptable carriers. The compositions can contain other ingredients conventional in cosmetics including perfumes, estrogen, vitamins A, C or E, alpha-hydroxy or alpha-keto acids such as pyruvic, lactic or glycolic acids, lanolin, vaseline, aloe vera, methyl or propyl paraben, pigments and the like.

The effective amount of the PAIs in the compositions used to treat dermatologic conditions or diseases can vary depending on such factors as condition of the skin, age of the skin, the particular PAI or degree of the purity of the PAIs employed, the type of formulation and carrier ingredients used, frequency of administration, overall health of the individual being treated and the like. The precise amount for any particular patient use can be determined by those of skill in the pharmaceutical art taking into consideration these factors and the present disclosure. Preferably the composition is administered in at least two doses and no more than about six doses per day, or less when a sustained or delayed release form is used.

The compositions for topical administration usually contain from about 0.0001% to about 90% by weight of the PAIs compared to the total weight of the composition, preferably from about 0.5% to

about 20% by weight of the PAIs to composition, and especially from about 2% to about 5% by weight of the PAIs to the composition.

The topical composition is administered by applying a coating or layer to the skin or mucosal area desired to be treated. As a practical matter of convenience, the applied material is rubbed into the area. Applications need not be rubbed into the skin and the layer or coating can be left on the skin overnight.

The present invention provides compositions suitable for transdermal administration including, but not limited to, pharmaceutically acceptable lotions, suspensions, oils, creams, ointments, rinses, gels and liposomal carriers suspended in a suitable vehicle in which a therapeutically effective amount of PAIs has been admixed. Such compositions are applied directly to the skin or incorporated into a protective carrier such as a transdermal device (so-called "patch"). Examples of suitable creams, ointments etc. can be found, for instance, in the Physician's Desk Reference. Examples of suitable transdermal devices are described, for instance, in U.S. Pat. No. 4,818,540 (Chien et al.).

The present invention includes compositions of PAIs suitable for parenteral administration including, but not limited to, pharmaceutically acceptable sterile isotonic solutions. Such solutions include, but are not limited to, saline and phosphate buffered saline for intravenous, intramuscular, intraperitoneal or subcutaneous injection of PAIs.

The present invention includes compositions of PAIs suitable for gastrointestinal administration including, but not limited to, pharmaceutically acceptable powders, pills or liquids for ingestion and suppositories for rectal administration.

The present invention includes compositions of PAIs suitable for transbronchial and transalveolar administration including, but not limited to, various types of pharmaceutically acceptable aerosols for inhalation. An example of a drug administered in the form of an aerosol is pentamidine which is administered to AIDS patients by inhalation to prevent pneumonia caused by Pneumocystis carnii.

The present invention further encompasses devices suitable for transbronchial and transalveolar administration of PAIs. Such devices include, but are not limited to, atomizers and vaporizers. The present invention also includes devices for electrical or direct injection. Electrical injection, or iontophoresis, is the process of using a small electrical current to drive charged elements, compounds and drugs through the skin for the purpose of delivering the therapeutic compound to the local tissues or to the whole body without breaking the skin.

The present invention further encompasses solutions suitable for storage of organs prior to transplantation. Suitable solutions are described in Chien et al. (1993) "Hibernation Induction Trigger for Organ Preservation" in Medical Intelligence Unit, R.G. Landes Co. Austin, Tex. PAIs ca be used, for instance, to replace much more impure soy preparations currently in use (e.g., Soyacal).

The above-mentioned compositions are meant to describe, but not limit, the compositions suitable for administering the PAIs of the invention. The methods of producing the various compositions are within the ability of one skilled in the art and are not described in detail here.

The methods of producing suitable devices for injection, topical application, atomizers and vaporizers are known in the art and will not be described in detail.

The invention further provides methods of treating apoptosis comprising administering an amount of

the PAIs effective to inhibit apoptosis. Various apoptosis-related indications can be treated by the method including, but not limited to, dermatological effects of aging, disorders and diseases, immunosuppression, gastrointestinal perturbations, cardiovascular disorders, rejection of tissue transplantation, and Alzheimer's disease.

It has now been found that PAIs may be topically applied to the skin to treat a variety of dermatologic conditions. These conditions include, but are not limited to, wrinkling or sagging due to age and/or photodamage, psoriasis. The present invention thus encompasses methods of treating dermatological conditions. Furthermore, baldness may be caused by apoptosis of the cells of the hair follicles. Therefore, the PAIs would be suitable for use in topical treatment of the skin to prevent continued hair loss.

As discussed above, these conditions are preferably treated by topical application of a composition comprising an effective amount of PAIs. An effective amount of PAI is one which ameliorates or diminishes the symptoms of the dermatologic conditions. Preferably, the treatment results in resolution of the dermatologic condition or restoration of normal skin function; however, any amelioration or lessening of symptoms is encompassed by the invention.

Immunosuppression related disorders are caused by a variety of stimuli which include, but are not limited to, viruses including, but not limited to, HIV, chemotherapeutic agents, and radiation. These stimuli trigger apoptosis in a variety of disorders, including, but not limited to, those of the digestive tract tissues and associated gastrointestinal perturbations.

Gastrointestinal perturbations include, but are not limited to, damage to the lining of the gut, severe chronic ulcers, colitis, radiation induced damage, chemotherapy induced damage, and the perturbation of the gastrointestinal tract caused by parasites, and diarrhea from any other cause. Various viral and bacterial infections are known to result in gastrointestinal perturbations; the PAIs are also suitable for use in treatment of the side effects associated with these infections. PAIs are particularly suited for use in ameliorating the gastrointestinal disturbances associated with chemotherapy. As shown in the Examples presented below, rats treated with methotrexate and various PAIs suffered less feeding problems and had none of the diarrhea found in the control animals. Thus, PAIs are suitable for use not only in preventing the diarrhea associated with chemotherapy but also the nausea.

The PAIs are particularly suited to treatment of various gastrointestinal conditions in animals, particularly cattle. Such conditions, particularly diarrhea, account for the loss of many calves. Treatment of gastrointestinal conditions is preferably by gastrointestinal administration. In the case of cattle, an effective amount of the PAIs can be conveniently mixed in with the feed. In humans, administration can be by any method known in the art of gastrointestinal administration.

In addition, the PAIs can be administered to immunodeficient patients, particularly HIV-positive patients, to prevent or at least mitigate apoptotic death of T cells associated with the condition, which results in the exacerbation of immunodeficiencies as seen in patients with full blown AIDS. Preferably, administration of PAIs to such patients is parenterally, but may also be transdermal or gastrointestinally.

The PAIs can also be administered to treat apoptosis associated with reperfusion damage involved in a variety of conditions, including, but not limited to, coronary artery obstruction; cerebral infarction; spinal/head trauma and concomitant severe paralysis; reperfusion damage due to other insults such as frostbite; and any indication previously thought to be treatable by superoxide dismutase (SOD). For

review of the effect of oxygen radicals in heart disease, see Singal (1988) "Oxygen Radicals in the Pathophysiology of Heart Disease" Kluwer Academic Publishers, MA, USA.

Myocardial and cerebral infarctions are caused generally by a sudden insufficiency of arterial or venous blood supply due to emboli, thrombi, or pressure that produces a macroscopic area of necrosis; the heart, brain, spleen, kidney, intestine, lung and testes are likely to be affected. Apoptosis occurs to tissue surrounding the infarct upon reperfusion of blood to the area; thus, PAIs are effective if administered at the onset of the infarct, during reperfusion, or shortly thereafter.

Thus, the invention includes methods of treating apoptosis associated with reperfusion comprising administering a therapeutically effective amount of at least one PAI to a patient in need of such therapy.

The invention further encompasses a method of reducing the apoptosis and reperfusion damage associated with myocardial and cerebral infarctions for patients with a high risk of heart attack and stroke by administering a therapeutically effective amount of at least one PAI to a patient in need of such therapy.

Preferably, treatment of reperfusion damage is by parenteral administration of the compositions of the invention. Any other suitable method may be used, however, for instance, direct cardiac injection in the case of myocardial infarct. Devices for such injection are known in the art, for instance the Aboject cardiac syringe.

The invention further provides methods of limiting and preventing apoptosis in cells during the culture or maintenance of mammalian organs, tissues, and cells by the addition of an effective amount of PAIs to any media or solutions used in the art of culturing or maintaining mammalian organs, tissues, and cells.

The invention further encompasses media and solutions known in the art of culturing and maintaining mammalian organs, tissues and cells, which comprise an amount of at least one PAI effective to limit or prevent apoptosis of the cells in culture.

These aspects of the invention encompass mammalian cell culture media comprising an effective amount of at least one PAI and the use of such media to limit or prevent apoptosis in mammalian cell culture. PAIs have been found to limit or prevent apoptosis under circumstances in which cells are subjected to mild traumas which would normally stimulate apoptosis. Such traumas may include, but are not limited to, low level irradiation, thawing of frozen cell stocks, rapid changes in the temperature, pH, osmolarity, or ion concentration of culture media, prolonged exposure to non-optimal temperature, pH, osmolarity, or ion concentration of the culture media, exposure to cytotoxins, disassociation of cells from an intact tissue in the preparation of primary cell cultures, serum deprivation (or growth in serum-free media).

Thus the invention encompasses compositions comprising tissue culture medium and an effective amount of at least one PAI. Serum-free media to which PAIs may be added as anti-apoptotic media supplements include, but are not limited to, AIM V.RTM. Media, Neuman and Tytell's Serumless Media, Trowell's T8 Media, Waymouth's MB 752/1 and 705/1 Media, and Williams' Media E. In addition to serum-free media, suitable mammalian cell culture media to which PAIs may be added as anti-apoptotic media supplements include, but are not limited to, Basal Eagle's Media, Fischer's Media, McCoy's Media, Media 199, RPMI Media 1630 and 1640, Media based on F-10 & F-12 Nutrient Mixtures, Leibovitz's L-15 Media, Glasgow Minimum Essential Media, and Dulbecco's

Modified Eagle Media. Mammalian cell culture media to which PAIs may be added further comprise any media supplement known in the art, including but not limited to, sugars, vitamins, hormones, metalloproteins, antibiotics, antimycotics, growth factors, lipoproteins and sera.

The invention further encompasses solutions for maintaining mammalian organs prior to transplantation, which comprise an effective amount of at least one PAI, and the use of such solutions to limit or prevent apoptosis in such mammalian organs during their surgical removal and handling prior to transplantation. In all cases concentrations of PAIs required to limit or prevent apoptosis can be determined empirically by one skilled in the art by methods like those found in Examples 2, 3 and 4, as well as other methods known in the art.

It has also been found that the PAI fractions above a certain concentration can form micelles in solution. The invention thus includes compositions comprising micelles.

The following examples are provided to illustrate but not limit the invention.

EXAMPLE 1

PAI Isolation and Purification

Approximately 100 g of commercially available soybean flour (Sigma Chemical Co. St. Louis, Mo. USA and Central Soya, Archer Daniel Midlands) was suspended in 500 ml of 70% acetone and stirred at room temperature for 30 minutes. The delipidated soybean flour was recovered by centrifugation at 1,500 g for 10 minutes. This material was resuspended in 1 l of 50% ethanol and stirred at room temperature for 30 minutes. The supernatant, the aqueous retentate, was reclaimed by centrifugation at 1,500 g for 10 minutes.

The aqueous retentate was concentrated by ultrafiltration and the ethanol was removed by diafiltration over a 10 kD membrane (Amicon Beverly Mass. USA). This material was then loaded directly onto Sepharose S100HR (Pharmacia Biotechnology, Inc. Piscataway, N.J., USA) equilibrated in 10 mM ammonium bicarbonate. The peak of A.sub.280 absorbing material eluted in the void volume and was pooled and lyophilized. The freeze-dried high molecular weight material was extracted into a single phase mixture of chloroform:methanol:water (3:8:4) by adding the single phase mixture to the dried material and mixing at room temperature for 30 minutes. The mixture was then centrifuged to remove the insoluble material. The insoluble material yielded a lipid/glycolipid fraction which retained PAI activity. This fraction has been termed the L/G fraction. The carbohydrate composition of the L/G fraction consists of arabinose and galactose in a 3:2 ratio with fucose, rhamnose, glucosamine, glucose and mannose all present as minor constituents. The carbohydrate composition was determined as described in Example 5.

The L/G fraction can be further separated on the basis of its solubility in a mixture of chloroform:methanol (80:20) and chromatography on silica (Silicic Acid 100 mesh, Mallinckrodt Chemical, Inc. KY). The silica chromatography is resolved in methanol to yield an active fraction (SiMe).

For a detailed summary of the physical and chemical characteristics of the soy flour extract at various stages of purification see Table 1, where ND stands for "none detected".

In Table 1, the activities and physical characteristics of the products of four stages of purification were determined. These four stages were: aqueous retentate; 70% acetone extract; 50% ethanol

extract of the 70% acetone pellet; and the high molecular weight fraction purified by size exclusion gel filtration chromatography from the 50% ethanol fraction. Protein yield is expressed as protein recovered per gram dry weight of starting material, as measured by the Bradford assay procedure (BioRad Laboratories). Anti-apoptotic activity is expressed as the calculated concentration of material (.mu.g/ml of media) required to save 50% of the cells released on serum free treatment as described in Example 2. Trypsin inhibition is expressed in relative units per .mu.g of protein in the sample. A relative unit (U) was defined as the amount of inhibitory activity which decreases by 50% the initial rate of hydrolysis of a 100 .mu.M substrate by 2 .mu.g of trypsin in a total volume of 1 ml. Absorbance values at 260 and 280 nm are expressed per gram of starting material in a 1 ml cell. This gives an indication of relative protein and nucleic acid concentrations present. The ratio of 260/280 was used to estimate the amount of nucleic acid present as described in Dawson et al., Data for Biochemical Research, Third Edition, 1990 published Oxford Science Publications.

TABLE 1
Physical Characterization of Soy PAI Extract at Various Stages of Purification

	Protein Concentration	Anti-apoptotic Activity	Trypsin Inhibition	Nucleic Acid A280	A260	Acid
Soy Flour Water Extract	196 mg/gm	Inhibitory	0.561 U/.mu.g	116/gm	134/gm	2.4%
Soy Flour 70% Acetone Extract	140 .mu.g/gm	Inhibitory	ND	4.5/gm	7.2/gm	11%
Soy Flour ND Acetone Pellet 50% Ethanol Extract (AcE)	310 .mu.g/gm	11 .mu.g/ml	177 U/.mu.g	32/gm	36/gm	
Soy Flour ND Acetone Pellet 50% Ethanol Gel Filtration Pool (FAcE)	62 .mu.g/gm	0.14 .mu.g/ml	3.5 U/.mu.g	10/gm	12/gm	
Soy Flour Acetone Pellet 50% Ethanol Gel Filtration Pool Organic Extract (L/G)	20 .mu.g/gm	45 ng/ml	ND	8.4/gm	9.1/gm	ND
Soy Flour Acetone Pellet 50% Ethanol Gel Filtration Pool Organic Extract Silicic acid Methanol	0.9 .mu.g/gm	12 ng/ml	ND	6.55/gm	7.7/gm	ND

Eluate
(SiMe)

EXAMPLE 2

Apoptosis Assay with C3H 10T1/2 Cells

In order to determine the apoptotic activity of the PAIs, the following experiment was performed. The cell assay is described in PCT Publication No. WO 9425621. Briefly, the cells, C3H 10T1/2 clone 8, were assayed at confluence (FIG. 1), during exponential growth phase when cell cycle position is randomly distributed with no cells arrested in G.sub.o (FIGS. 2 and 3), and in quiescence (FIG. 4). Exponential growth phase was assured by seeding at 2000 cells per 1 ml (5 ml for a 60 mm culture plate) five days prior to the beginning of the experiment. At T=0, cultures were transferred to serum-free medium, as an apoptosis stimulus, and seed extracts were added. Controls included 10.sup.-7 M 12-O-tetradecanoyl phorbol-13-acetate (TPA) to ensure the responsiveness of the cell culture. The PAI samples prepared by extraction with ethanol and by gel filtration were added to serum free medium at 0.1 g dry weight equivalents and sterile filtered prior to addition to the cultures. Assays were performed in triplicate or quadruplicate. Analyses of the cell responses were made after between 22 and 28 hours of serum deprivation and/or treatment with soy flour derived PAIs. Two assays were performed on each cell culture plate consisting of differential cell counts.

1. All non-adherent or loosely adherent cells were removed from the culture dish and counted by appropriate techniques, typically counting by electric particle counting instrument. These are the apoptotic cells, the serum deprived released cells (SDR), released by the action of cultivation in serum-free medium. Approximately 95% of these released cells are apoptotic as shown by both ultrastructure analysis and DNA fragmentation analysis.

2. The remaining adherent cells (ADH) are exposed to a buffered, typically pH 7.3, balanced salt solution such as Hanks Balanced Salt Solution without calcium and magnesium salts containing 0.05% trypsin and 0.53 mM ethylenediaminetetraacetic acid (EDTA). Each culture is incubated at either room temperature or 37.degree. C. on a rocking platform to ensure uniform distribution of the trypsin reagent over the culture surface. After a standardized period of time, typically 10 minutes, the released cells are removed from each culture dish and measured by the same means as described above, typically electronic particle counting. This ADH cell count is comprised of both trypsin resistant and trypsin sensitive cells as described in PCT Publication No. WO 9425621.

The results obtained from the apoptosis cell assays are presented in FIGS. 1, 2, 3 and 4. In FIG. 1 the percentage of cells having undergone apoptosis (SDR) and adherent cells (ADH) are presented separately. The Data in FIG. 1 demonstrate that the PAIs are effective in reducing apoptosis in confluent cells, as compared with the Basal Medium Eagle (BME), serum-deprived control.

In FIG. 2, the results are presented as a percentage of adherent cells in the samples treated with PAIs normalized for the number of adherent cells in the serum free control sample without PAIs. In other words, the percentage of cells saved from apoptosis by treatment with PAIs. The data presented in FIG. 2 demonstrate that soy PAIs have a concentration-dependent anti-apoptotic effect on C3H 10T1/2 cells in exponential growth phase.

In FIG. 3, the anti-apoptotic activity for PAIs after extraction with 50% ethanol (right side) and PAIs

which have been further purified by size exclusion gel filtration chromatography (left side) is presented. The results in FIG. 3 are expressed as the percentage of cells saved from apoptosis (SDR cells converted to ADH cells) by treatment with PAIs as compared with the no PAI serum free control samples, all of which is expressed as a function of trypsin inhibitory units. The data presented in FIG. 3 demonstrate that when the concentration of Bowman-Birk inhibitors (as measured by trypsin inhibition) is reduced by size exclusion gel filtration chromatography, the anti-apoptotic activity of PAIs is maintained. Thus, the anti-apoptotic activity of the PAI preparations is not due to the presence of Bowman-Birk inhibitors.

In FIG. 4, the anti-apoptotic activity of various concentrations of soy PAIs on quiescent C3H 10T1/2 cells treated with cycloheximide is presented. Quiescent cells are those which no longer respond to serum deprivation by entering apoptosis. Rather, apoptosis is stimulated in these cells by the addition of 10 .mu.g/ml cycloheximide in C3H 10T1/2. Typically, these cells become confluent after about one week in culture and quiescent after about two weeks in culture. The results in FIG. 4 are expressed as viable cells remaining (ADH) after a given treatment. The data in FIG. 4 demonstrate that soy PAIs have a small, but significant anti-apoptotic effect on quiescent C3H 10T1/2 cells.

EXAMPLE 3

Apoptosis Assay with Neonatal Rat Cardiac Myocytes

Myocytes were prepared from hearts of day-old rats as described in Circulation Research 56:884-894, 1985. In brief, the individual cells were obtained by brief, alternating cycles of room temperature trypsinization and mechanical disaggregation. The cells were collected, washed, and resuspended in MEM, 5% fetal bovine serum and 50 U/ml penicillin-G. To reduce contamination by non-myocytes, the cells were pre-plated for 30 minutes. The non-adherent cardiac myocytes were removed from the culture dish, counted on a hemocytometer, and resuspended in medium to a concentration of 600,000 viable cells/ml. The cell suspension was distributed into different culture dishes and incubated in a 37.degree. C., 5% CO.sub.2 incubator for 16-24 hours. The yield was 3-5.times.10.sup.6 cells/heart and viable cells were >90% by trypan blue staining.

On the first day of culture, the cells were rinsed with Minimal Eagle Medium (MEN) several times to remove debris and non-adherent cells. They were replenished with serum supplemented media as above. The myocytes were challenged with different conditions in RPMI 1640 medium the next day. The results obtained are presented in Table 2, where PAI 1 x represents the material obtained from 0.1 g of soy flour starting material.

TABLE 2

Neonatal Cardiomyocytes

	Beating Rate	Cell Number
Serum-free	+	11606
Conditioned Media	+++++	5128
PAI 1 x	+++	15062

The results obtained indicate that the PAI fraction is capable of preserving the well-being of cells in the presence of an apoptosis-inducing insult.

EXAMPLE 4

Determination of Carbohydrate Composition of PAI

In order to determine whether PAIs contain carbohydrate, the L/G fraction of PAIs were exposed to various conditions and the resulting carbohydrate residues were assayed. The PAIs were obtained from Sigma Soy flour Lot No. 103H0820 treated to obtain PAIs as described in Example 1. The free monosaccharides in untreated PAIs were determined by HPLC on Dionex Carbopac.TM. PA1 in 16 mM NaOH according to the method described in Dionex Document No. 034441. The results obtained are presented in Table 3. The sample was then hydrolyzed with 2N TFA for four hours at 100.degree. C. as described by Hardy and Townsend (1994) Meth. Enzymol. 230:208-225. The results obtained are presented in Table 4. The sample was further hydrolyzed with 6 N HCl for 6 hours at 100.degree. C. as determined by the method described by Hardy and Townsend (1994). The results obtained are depicted in FIG. 5.

TABLE 3

Monosaccharides Detected in PAI

Monosaccharide	ng/25 .mu.g PAI	ng/mg PAI
Arabinose	1.098	45.9
Glucose	0.058	2.36
TOTAL	1.157	48.26

TABLE 3

Monosaccharides Detected in PAI

Monosaccharide	ng/25 .mu.g PAI	ng/mg PAI
Arabinose	1.098	45.9
Glucose	0.058	2.36
TOTAL	1.157	48.26

EXAMPLE 5

Use of PAIs to Prevent Chemotherapy Induced Gastrointestinal Disorders

In order to determine the in vivo activity of the PAIs, the following animal experiments were performed. In Examples 6 and 7 the animal tests were performed essentially as described in Funk and Baker (1991) J. Nutr. 121:1684-1692; and Funk and Baker (1991) J. Nutr. 121:1673-1683. Briefly, male Sprague-Dawley rats were used to determine if isolated AcE and L/G obtained from sly flour as described in Example 1 could alleviate methotrexate (MTX) toxicity. The rats were housed in individual, wire-bottom stainless steel cages and were adapted to their respective diets for 7 days prior to injection of MTX and remained on the same diets for 7 days after injection. Diets fed were semipurified rat food with the following additions:

1. casein

2. casein and soy concentrate (10 g and 10 g)

3. casein and soy flour (10 g and 10 g)

4. casein and AcE

5. casein and L/G

AcE and L/G were used at concentrations equal to that extracted from the soy starting material.

Records of rat weight and food intake were kept during the preinjection period. Rats were injected IP with 20 mg/kg MTX. During the 7 day postinjection period, rat weight, food intake and incidence of diarrhea were recorded. Rat weight and food intake data were analyzed using the nonparametric Kruskal-Wallis test and post-hoc comparison. The P value was adjusted for multiple comparisons by dividing 0.05 by the number of comparisons made (10). A nonparametric test was used because variances between groups were not homogeneous and thus assumptions for analysis of variances were not met. Food intake following MTX injection was expressed as a percentage of the average intake 3 days prior to injection for each animal. Thus, each animal served as its own control. Only days 3, 4, 5, and 6 post MTX injection were analyzed statistically. The reason for this is that days 3 and 4 are when toxicity is most severe and days 5 and 6 are when recovery begins. Diarrhea data were analyzed using both the Fisher's Exact Test and loglinear analysis.

Results showed that soy concentrate and soy flour starting material as well as both the AcE and L/G were capable of improving food intake following MTX injection (Table 5 and FIG. 7). On day 3 food intake for rats consuming soy flour and soy concentrate was statistically greater than for those consuming casein alone or casein with L/G. Rats consuming casein with AcE were intermediate in food intake on day 3 and statistically similar to all groups except those consuming soy flour. Day 4 showed an identical pattern except since food intake for rats consuming L/G rose slightly as compared with day 3, these rats were no longer statistically different from rats consuming soy concentrate although numerically food intake remained substantially lower. Recovery was evident on days 5 and 6 and food intake was statistically similar among all groups. Weight change (Table 6) reflected patterns observed in food intake which is expected. Rats consuming soy concentrate and soy flour gained a substantial amount of weight during the first 4 days following MTX injection. Rats consuming AcE, L/G or casein alone gained less weight and those consuming casein gained statistically less than those fed soy concentrate or soy flour. Differences in incidence of diarrhea were not statistically different but the pattern of diarrhea was consistent with food intake and weight change (Table 6). Rats consuming soy concentrate or soy flour had no diarrhea while a slight amount of diarrhea was present in rats consuming AcE (10%) and a moderate amount of diarrhea was present in those consuming L/G or casein alone (30-40%).

In conclusion, this experiment showed that soy concentrate and soy flour offered the best protection of the components tested. Casein with AcE appeared to be intermediate and superior to casein alone or L/G as evidenced by better maintenance of food intake and weight and lower incidence of diarrhea. This result indicates that compounds isolated from soy can provide protection against MTX toxicity. In this experiment, the L/G fraction at this concentration did not appear to provide protection. However, Example 7 shows that increased concentrations of L/G are effective.

TABLE 5

Effect of Diet and Methotrexate on Food Intake.sup.1

Diet	Pretreatment Food Intake.sup.2 (g/day)	Posttreatment (%).sup.3 Food Intake day 3	day 4	day 5	day 6
Casein	18.4 .+-. 0.6	34.1 .+-. 11.0.sup.a	39.6 .+-. 13.0.sup.a	83.0 .+-. 12.5	108.9 .+-. 2.7
Soy Concentrate-Casein (50/50)	18.4 .+-. 0.5	94.3 .+-. 6.5.sup.bc	94.8 .+-. 5.1.sup.bc	98.9 .+-. 2.5	101.8 .+-. 3.3
Soy Flour-Casein (50/50)	17.8 .+-. 0.4	99.0 .+-. 4.6.sup.c	99.1 .+-. 2.0.sup.c	98.2 .+-. 2.3	102.1 .+-. 3.5
Casein-AcE	18.5 .+-. 0.4	63.6 .+-. 9.3.sup.ab	68.3 .+-. 10.3.sup.ab	88.6 .+-. 6.9	99.5 .+-. 4.0

Casein-L/G 19.0 .+-. 0.8 46.3 .+-. 11.7.sup.a 53.9 .+-. 14.2.sup.ab 75.3 .+-. 13.1 96.4 .+-. 11.0

.sup.1 Values are means .+-. standard error of the mean for ten male rats. Methotrexate was injected IP following a 7-day adaptation period. Values in columns with unlike superscripts differ (P .ltoreq. 0.05, Kruskal-Wallis test and post hoc comparison).

.sup.2 Pretreatment food intake represents the mean of the 3 day period prior to the administration of MTX.

.sup.3 Posttreatment intake represents the % of pretreatment intake.

TABLE 6

Effect of Diet and Methotrexate on Rat Weight.sup.1

Diet	Average Pretreatment Weight.sup.2 (g)	Weight Change day 0-4 (g)	Weight Change day 4-6 (g)	Incidence of Diarrhea (%)
Casein	230.0 .+-. 4.7	1.2 .+-. 5.3.sup.a	16.3 .+-. 1.5	30
Soy Concentrate-Casein (50/50)	227.2 .+-. 4.7	22.1 .+-. 1.5.sup.b	9.7 .+-. 1.6	0
Soy Flour-Casein (50/50)	227.2 .+-. 4.4	22.7 .+-. 1.6.sup.b	10.1 .+-. 1.0	0
Casein-AcE	230.3 .+-. 3.2	11.7 .+-. 4.1.sup.ab	13.5 .+-. 2.1	10
Casein-L/G	232.9 .+-. 5.1	6.9 .+-. 5.6.sup.ab	9.8 .+-. 4.5	40

.sup.1 Values are means .+-. standard error of the mean for ten male rats. Methotrexate was injected IP following a 7-day adaptation period. Values in columns with unlike superscripts differ (P.ltoreq. 0.05, Kruskal-Wallis test and post hoc comparison).

.sup.2 Pretreatment weight indicates the average weight on the day of injection.

EXAMPLE 6

Use of PAIs to Prevent Chemotherapy Induced Gastrointestinal Disorders

Male Sprague-Dawley rats were used to determine if graded levels of isolated soy fractions (AcE, L/G and MAcE) could alleviate methotrexate (MTX) toxicity. Animals were housed in individual, wire-bottom stainless steel cages. Rats were adapted to their respective diets for 7 days prior to injection of MTX and remained on the same diets for 7 days after injection. Diets fed were semipurified and casein with additions as follows:

1. No additions

2. AcE (100 mg/20 g casein; 1.times.)

3. AcE (300 mg/20 g casein; 3.times.)

4. AcE (1000 mg/20 g casein; 10.times.)

5. L/G (10 mg/20 g casein; 1.times.)

6. L/G (30 mg/20 g casein; 3.times.)

7. L/G (100 mg/20 g casein; 10.times.)

8. MAcE (100 mg/20 g casein; 1.times.)

9. MAcE (300 mg/20 g casein; 3.times.)

10. MAcE (1000 mg/20 g casein; 10.times.)

11. MAcE (3000 mg/20 g casein; 30.times.)

Note that MAcE is soy molasses extracted as for soy flour to obtain AcE. Each diet group contained 8 rats except for the group receiving casein with no added compound which contained 10 rats. Records of rat weight and food intake were kept during the preinjection period. Rats were injected IP with 20 mg/kg MTX. During the 7 day postinjection period, rat weight, food intake and incidence of diarrhea were recorded. Food intake for various groups is depicted in FIGS. 8-10. Incidence of diarrhea is depicted in FIG. 11. Rat food intake for the entire group is depicted in FIG. 12. Rat weight and food intake data were analyzed by ANOVA using a factorial arrangement of treatments to test the main effects of compound and dose and the possible interaction between compound and dose. Factorial analysis was done using only the treatment groups with the 1.times., 3.times. and 10.times. doses of each of the compounds. In addition t-tests were used to determine differences between the 10.times. level of each compound and the diet containing only casein. Food intake following MTX injection was expressed as a percentage of the average intake 3 days prior to injection for each animal. Thus, each animal served as its own control. Only days 3, 4, 5 and 6 post MTX injection were analyzed statistically. The reason for this is that days 3 and 4 are when toxicity is most severe and days 5 and 6 are when recovery begins. Diarrhea data were analyzed using Fisher's Exact Test. Only the 10.times. levels for each of the compounds were analyzed statistically against casein for differences in diarrhea incidence. FIG. 12. The reason for this is that Fisher's test is a conservative test. When multiple comparisons are done the error rate must be adjusted. In order to increase the chances of statistical significance, only those comparisons were done where the best response for each of the compounds had been realized as evidenced by food intake and weight change data.

Results of the factorial analysis of food intake and weight change are presented in Tables 7 and 8. The results showed that the AcE compound was the most effective at alleviating MTX toxicity. Food intake was greater for all AcE groups combined than for both of the other groups on day 3 following MTX dosing and remained greater than those consuming MAcE on day 4 ($P<0.05$). Decreased toxicity in rats consuming AcE as compared with MAcE was also reflected in weight patterns as those consuming AcE gained more weight during the first four days postdosing than those consuming MAcE ($P<0.05$). Improvements in intake and maintenance of weight were seen with increasing levels of each of the compounds with the exception of the 30.times. level of MAcE, although this was not statistically significant. The level of each compound where response was the best was 10.times.. In comparing the 10.times. level of each of the compounds against casein alone, AcE was statistically greater on days 3, 4 and 5 postdosing ($P<0.05$). The pattern of diarrhea was consistent with the food intake results. Fifty percent of the animals consuming casein developed diarrhea. No animals consuming the 10.times. level of the compounds developed diarrhea which was statistically less than those consuming casein alone ($P=0.088$). All other groups experienced some diarrhea with the exception of those consuming the 3.times. level of AcE.

In conclusion, for the compounds tested, AcE was the best at alleviating MTX toxicity. L/G and MAcE positively affected MTX toxicity as evidenced by decreased incidence of diarrhea as compared with casein alone and statistically nonsignificant improvement in food intake and weight

maintenance. It is possible that higher levels of AcE and L/G may provide additional protection. The 30.times. level of MAcE proved to be ineffective and closely resembled casein alone. Therefore, it appears that once a threshold is reached higher levels are detrimental. It is possible that MAcE may be more effective at a dose somewhere between the 10.times. and 30.times. levels tested in this experiment.

TABLE 7

Effect of Diet and Methotrexate on Food Intake.sup.1

Diet	n	Pretreatment Food Intake (g/day)	Food Intake Posttreatment (%) day 3.sup.2,3	day 4.sup.3,4	day 5.sup.3	day 6
1.	10	18.5 .+-. 0.5	39.5 .+-. 11.5	33.5 .+-. 11.7	58.9 .+-. 12.4	96.3 .+-. 5.9
2.	8	19.7 .+-. 0.6	57.6 .+-. 12.8	52.7 .+-. 16.6	71.5 .+-. 11.2	93.0 .+-. 4.8
3.	8	16.2 .+-. 0.4	61.4 .+-. 7.0	71.3 .+-. 11.3	92.6 .+-. 6.0	105.6 .+-. 4.7
4.	8	16.7 .+-. 0.4	78.2 .+-. 9.2	76.7 .+-. 11.0	90.3 .+-. 6.7	97.8 .+-. 4.3
5.	8	17.9 .+-. 0.4	40.0 .+-. 8.4	45.9 .+-. 11.4	80.3 .+-. 11.4	105.7 .+-. 3.6
6.	8	18.3 .+-. 0.7	46.0 .+-. 9.8	50.7 .+-. 12.8	79.6 .+-. 12.0	105.2 .+-. 5.3
7.	8	17.3 .+-. 0.6	60.8 .+-. 9.9	61.9 .+-. 12.8	79.2 .+-. 11.8	103.3 .+-. 3.9
8.	8	18.5 .+-. 0.7	40.8 .+-. 10.2	34.5 .+-. 9.4	59.1 .+-. 9.8	100.2 .+-. 4.8
9.	8	17.2 .+-. 0.5	38.0 .+-. 8.4	45.4 .+-. 10.6	82.2 .+-. 11.7	100.4 .+-. 6.4
10.	8	16.9 .+-. 0.4	50.4 .+-. 5.8	52.2 .+-. 9.4	70.4 .+-. 13.9	89.3 .+-. 12.0
11.	8	16.4 .+-. 0.4	30.0 .+-. 6.0	33.9 .+-. 9.8	58.1 .+-. 9.1	110.4 .+-. 4.0

Footnotes:

.sup.1 Values are means .+-. standard error of the mean for male rats. Methotrexate was injected IP following a 7-day adaptation period. Pretreatment food intake represents the mean of the 3-day period prior to the administration of methotrexate. Posttreatment intake represents the % of pretreatment intake.

.sup.2 The casein-AcE groups (2-4) maintained a better posttreatment intake than casein-L/G (5-6) and casein-MAcE (8/11) groups (P < 0.05, ANOVA and Student Newmans-Keuls tests following factorial analysis).

.sup.3 Casein-AcE (10.times.) (3) animals had a greater posttreatment intake than casein (P < 0.05), t-test).

.sup.4 The casein-AcE groups maintained a better posttreatment intake than the casein-MAcE groups (P < 0.05, ANOVA and Student Newmans-Keuls tests following factorial analysis).

TABLE 8

Effect of Diet and Methotrexate on Rat Weight and Diarrhea.sup.1

Diet	n	Average Pretreatment Weight (g)	Weight Change day 0-4.sup.2 (g)	Weight Change day 4-6 (g)	Incidence of Diarrhea.sup.3 (%)
1.	10	220.2 .+-. 5.5	-2.1 .+-. 1.8	13.2 .+-. 1.8	50
2.	8	233.5 .+-. 4.2	8.3 .+-. 5.6	11.1 .+-. 0.7	25
3.	8	224.7 .+-. 3.6	9.0 .+-. 3.0	10.8 .+-. 2.0	0
4.	8	227.3 .+-. 3.8	12.4 .+-. 5.4	10.6 .+-. 1.5	0
5.	8	232.6 .+-. 2.7	2.5 .+-. 3.6	14.3 .+-. 1.9	12.5
6.	8	225.5 .+-. 3.3	3.8 .+-. 3.9	12.2 .+-. 1.8	12.5
7.	8	232.6 .+-. 4.5	6.3 .+-. 5.0	11.3 .+-. 1.2	0

8.	8	234.1 .+-. 6.3	0.0 .+-. 2.3	13.5 .+-. 2.4	12.5
9.	8	226.1 .+-. 3.2	1.5 .+-. 4.9	11.6 .+-. 1.7	25
10.	8	235.2 .+-. 2.3	3.1 .+-. 3.2	7.9 .+-. 3.8	0
11.	8	220.6 .+-. 2.8	-2.3 .+-. 3.4	140.0 .+-. 1.2	25

Footnotes:

.sup.1 Values are means .+-. standard error of the mean for male rats. Methotrexate was injected IP following a 7-day adaptation period. Pretreatment weight indicates the average weight on the day of injection.

.sup.2 The casein-AcE groups (2-4) maintained overall weight better during acute toxicity than the casein-MAcE groups (8-11) (P < 0.05, ANOVA and Student Newmans-Keuls tests following factorial analysis). Casein-AcE (10.times.) (4) animals showed significantly less weight loss than casein (P < 0.05), t-test).

.sup.3 Animals consuming the 10.times. level of the compounds (4, 7, 10) had a significantly lower incidence of diarrhea than animals consuming casein (P < 0.088, Fisher's exact test for diarrhea).

EXAMPLE 7

Use of PAIs to Inhibit Apoptosis in Lymphocytes Obtained from an HIV-infected Patient

The L/G fraction of PAIs isolated from soy flour was tested for its ability to inhibit apoptosis in lymphocytes from an HIV-infected patient.

Peripheral blood monocytes (PBMCs) were obtained from the patient and isolated according to standard methods. The PBMCs were cultured at 2.times.10.sup.6 /well in 24-well plates (Costar.times.Cambridge, Mass.) for 72 hours at 37 C. and 5% CO.sub.2 containing 2 ml/well of RPMI 1640 with antibiotics and 10% hAB. Some cultures contained 10 .mu.g/ml Pokeweed Mitogen (PWM) (Sigma, St. Louis, Mo.). Suspensions of thymocytes were used immediately after removal or after culture in RPMI+10% fetal bovine serum with 5 .mu.M dexamethasone (DEX) (Sigma--St. Louis, Mo.) for 18 hours. The cells were exposed for three days to the L/G fractions at the concentrations indicated below where 0.5 gEQ is the fraction derived from 0.5 g starting weight of flour.

Lane #	PWM	L/G
1	-	None
2	-	Purified L/G - 0.5 gEQ/ml
3	-	Purified L/G - 0.05 gEQ/ml
4	-	Purified L/G - 0.005 gEQ/ml
5	+	None
6	+	Purified L/G - 0.5 gEQ/ml
7	+	Purified L/G - 0.05 gEQ/ml
8	+	Purified L/G - 0.005 gEQ/ml
9	-	Untreated rat thymocytes (negative control)
10	-	Dexamethasone-treated rat thymocytes (positive control)
11	-	123 bp DNA standards

DNA was extracted and gel electrophoresis was performed as described by Sambrook et al. Molecular Cloning--Laboratory Manuals, 2nd Ed. Cold Spring Harbor Laboratory Press, NY pp. 134-135, E3-E4 and E10-11. Briefly, cells harvested were pelleted by centrifugation and lysed in

400 .mu.l of 50 mM KCl, 10 mM Tris-HCl (pH 8), 1% NP-40, 1% Tween-20, and 0.5 mg/ml Proteinase K (Boehringer Mannheim, Indianapolis, Ind.) at 60 C. for 1 hour. After extraction with phenol-chloroform and recovery with ethanol, the DNA was run through a 1.5% agarose gel (SeaKem, Rockland, Me.) in 90 mM Tris-Borate, 2.5 mM EDTA, pH 8.3 at 30 to 50 V for approximately 4 hours. A 123 base pair ladder (GIBCO BRL, Gaithersburg, Md.) was used as the DNA standard (DS) markers. Gels were stained with 1 .mu.g/ml ethidium bromide (Molecular Probes, Eugene, Oreg.) and destained in distilled H.sub.2 O.

The results are shown in FIG. 13 where the lanes are as indicated above.

The results obtained indicate that significant DNA fragmentation was observed in the absence of PWM-stimulation (lane 1) and this fragmentation was almost completely inhibited in cultures that contained 0.5 gEQ of L/G (lane 2). Lower concentrations of L/G (0.05 gEQ and 0.005 gEQ) did not inhibit DNA fragmentation in the absence of PWM (lanes 3 and 4) or in the presence of PWM (lanes 7 and 8). The DNA fragmentation in the presence of PWM (lane 5) was increased in comparison to cultures without PWM (lane 1). A slight inhibition of DNA fragmentation in the presence of PWM was observed in the presence of 0.5 gEQ L/G (lane 6) in comparison to lane 5. Negative and positive controls (lanes 10 and 11) worked as expected.

EXAMPLE 8

Further Purification and Characterization of PAIs

Samples were purified by extraction as described in Example 1 then silica, diol and HPLC silica chromatography were performed and the products analyzed for chemical composition, molecular weight, and structure. From the silica HPLC, the flow through and five major peaks were observed.

The flow through contained lysophosphatidic acid as determined by NMR proton and carbon 13 analysis. Fatty acid analysis indicated a mixture of C16:0 and C18:2 (hexadecanoic and 9,12-octadecadienoic) in the ratio of 60:40 to 50:50 depending on the soy starting material.

Peak two was identified as phosphatidyl inositol by mass spectrometry, NMR, and co-migration with authentic standards on TLC analysis. In addition, fatty acid analysis demonstrated a similar ratio of C16:0 and C18:2 (hexadecanoic and 9,12-octadecadienoic) on each of the two possible positions in the ratio of 60:40 (this is the majority and is typical for soy phosphatidyl inositol) to 50:50 depending on the position of sample in the peak i.e. leading or trailing edge.

Peak three contains four identifiable fatty acids of the C16:0, C18:0 C18:1 and C18:2 varieties i.e., hexadecanoic, octadecanoic, cis-9-octadecenoic, and 9,12-octadecadienoic in the ratio of 40:5:10:45, the most active containing a ratio of 45:5:5:45. In addition, this peak contains an unidentified fatty acid component migrating at an elution time of 16.2 to 16.3 minutes; much later than 16:0 (at 10.8 minutes) and the 18:0, 18:1, and 18:2 that elute between 13.2 and 14.1 minutes. The unidentified moiety comprised from 50 to 68% of the total fatty acid present. Using mass spectrometry analysis, lysophosphatidyl inositol was identified, with both the 16:0 and 18:2 fatty acid varieties. This peak also contains phosphatidyl inositol with 16:0 and 18:2 fatty acid on the R1 and R2 positions. Three unidentified species with molecular weights of 861, 864 and 939-940 were also found.

Peak 3 has been designated "D" in that it has been found primarily in the soy flour extract. Peak 4, designated "B", has no anti-apoptotic activity.

Peak 5 has been designated "L" and is found primarily in the lecithin-derived material. Peak 5 contains two identifiable fatty acids of the C16:0, C18:0 varieties, i.e. hexadecanoic and octadecanoic, in the ratio of 75:25. In addition, this peak contains an unidentified fatty acid component migrating with an elution time of 19 to 22 minutes. The unidentified moiety comprises 66% of the total fatty acid present. Using mass spectrometry, phosphatidyl inositol was identified in the 16:0 and 18:0 fatty acid variety. Two unidentified species with molecular weights of 113 and 191 were also observed.

Fatty acids were analyzed as fatty acid methyl esters. The transesterification reagent was anhydrous HCl/MeOH prepared as described in Christie "HPLC and Lipids" (1987), and analyzed as described in Christie "Gas Chromatography and Lipids: a practical guide" (1989), both published by Oily Press Ltd. Dundee Scotland. To each sample, 300 uL of CH.sub.2 Cl.sub.2 and 700 uL of HCl/MeOH was added. Derivatization was done under nitrogen at room temperature for 18 hours. After that, 1 mL of water was added and the samples were extracted with 3.times.2 mL of hexane. The combined extractions were dried under a stream of nitrogen redissolved in 100 uL of hexane and transferred to GC-MS vials. Analyses of samples were performed on a Hewlett-Packard 5890 gas chromatograph with a Hewlett-Packard 5971 series mass selective detector as described in van den Berg et al. (1993) J. Lipid Res. 34:2005-2012.

Electrospray mass spectrometry (MS) was performed on a VG BloQ Triple quadrupole mass spectrometer with electrospray ionization in negative mode. The source temperature was 80.degree. C., the solvent was methanol or methanol with 0.05% ammonium acetate at 5 uL/min, and capillary voltage was 4.7 kV. Mass spectrometry is generally described in "Christie's Gas Chromatography and Lipids" (1989).

EXAMPLE 9

Anti-apoptotic Activity of Known Phospholipids

Known phospholipid compounds were assayed for anti-apoptotic activity on serum-deprived 10T1/2 cells as described in Example 2. All commercial samples were dissolved at a concentration of 10 mM (100.times. test concentration) in 1% bovine serum albumin (BSA), 0.5 mM calcium chloride, 0.5 mM magnesium chloride, at room temperature. All compounds were tested after pre-incubation in 1% BSA. The final concentration of BSA in the apoptosis was .ltoreq.0.01%. Pre-incubation with BSA or fraction "B," which is mainly PI, enhanced the anti-apoptotic activity of LPA (FIG. 14). All tested compounds were obtained from Sigma. The BSA was obtained from Boehringer Mannheim. The results obtained are presented in Table 9 and FIG. 15.

TABLE 9

COMPOUND	ACTIVITY
L-a-lysophosphatidic acid, oleoyl (C18:1, [cis]-9)	** ++
L-a-lysophosphatidyl-L-serine	++
L-a-lysophosphatidyl choline, Type VI	--
L-a-lysophosphatidyl inositol **	--
L-a-lysophosphatidyl ethanolamine, Type IV	0
L-a-phosphatidic acid, dioleoyl (C18:1, [cis]-9)	0
L-a-phosphatidyl-L-serine, from bovine brain	0
L-a-phosphatidyl choline Type V-EA	0
L-a-phosphatidyl inositol **	0

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    L-a-phosphatidyl ethanolamine, Type IV          0
Key: ++ anti-apoptotic activity
0  no activity
-- apoptotic or necrotic effect
** compounds identified by mass spec., present in active fractions
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Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the descriptions and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

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